Control of IFN- response via autocrine activation of the C5a complement receptors and the NLRP3 inflammasome within human T lymphocytes

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Control of IFN-γ response via autocrine activation of the C5a complement receptors and the NLRP3 inflammasome within human T lymphocytes

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Abstract

IL-1β is a pro-inflammatory cytokine critical for the protection against pathogens. The NLRP3 inflammasome mediates the maturation of IL-1β, however uncontrolled inflammasome activity contributes to the development of several diseases with strong impact on public health.

The complement system is involved in the direct elimination of pathogens and in shaping adaptive immune responses. Particularly, paracrine and/or autocrine signals mediated by the complement fragments C3a and C3b regulate the induction and contraction of human Th1 responses.

The function of C5a in T cells was still unexplored. Preliminary evidences supported a role for C5a in modulating the NLRP3 inflammasome.

This thesis investigates the potential function of autocrine complement C5a-mediated signals and the NLRP3 inflammasome within human $CD4^+$ T cells during Th1 effector responses.

This work demonstrates that intracellular C5 activation (driven by T cell receptor and CD46 signaling) and C5aR1 engagement induce ROS production. These events lead to T cell intrinsic NLRP3 inflammasome activation and IL-1β secretion, which support IFN-γ production and Th1 induction in an autocrine fashion. Surface-expressed C5aR2 negatively regulates this process. $CD4^+$ T cells from patients with cryopyrin associated periodic syndrome (CAPS), which have mutated, constitutively-active, NLRP3 inflammasome, had overactive *in vitro* Th1 responses, normalised by NLRP3 inhibition. In collaboration with Dr. Erin West (NIH, MD, USA), *in vivo* significance of NLRP3 inflammasome activity within T cells was shown using models of viral infection, colitis and graft versus host disease (GvHD). Together, these data indicate the requirement for intrinsic NLRP3 activation for normal Th1 induction in mouse and human $CD4^+$ T cells. This work resulted in the publication of a peer-reviewed original research article entitled "T helper 1 immunity requires complement-driven, NLRP3 inflammasome activity in $CD4^+$ T cells" in *Science* (Arbore *et al*., 2016), which is incorporated in this thesis.

Finally, in the last thesis chapter, it has been investigated whether optimal production of IFN-γ by $CD8^+$ T cells also relies on autocrine complement and NLRP3 inflammasome activities.

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Declaration

I declare that the experiments and the data herein reported derive from my own work and any effort has been made to indicate clearly the work performed by other laboratory members and external collaborators.

Publications arising from this work

Original publications:

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Abbreviations

AA: amino acid

AB: amyloid-β

AD: Alzheimer's disease

AIM2: absent in melanoma 2

AMD: age-related macular degeneration

AMP: adenosine monophosphate

AMPK: AMP-dependent protein kinase

APC: antigen presenting cell

ARRB: arrestin-β

ASC: apoptosis speck protein

ATP: adenosine triphosphate

BCR: B cell receptor

C1-INH: C1 esterase inhibitor

C2TA: class 2 transcription activator of the MHC

C3aR: C3a receptor

C4BP: C4b binding protein

C5aR1: C5a receptor 1

C5aR2: C5a receptor 2

cAMP: cyclic AMP

CAPS: cryopyrin associated periodic syndrome

CARD: caspase recruitment domain

CC: cholesterol crystals

CCR5: C-C chemokine receptor 5

CD: Crohn's disease

CD(n): cluster of differentiation (number)

CFSE: carboxyfluorescein succinimidyl ester

CINCA: chronic infantile neurologic cutaneous and articular syndrome

CLP: cecal ligation and puncture

CNS: central nervous system

CPPD: calcium pyrophosphate dehydrate

CR: complement receptor

CRP: C-reactive protein

CTL: cytotoxic T lymphocyte

CTSL: cathepsin L

CYT: cytoplasmic tail

D: diversity

DAF: decay accelerating factor

DAMP: danger-associated molecular pattern

DC: dendritic cell

DMSO: dimethylsulphoxide

DSS: dextran sulphate

EAE: experimental autoimmune encephalomyelitis

EP4: PGE2 receptor subtype 4

FACS: fluorescence-activated cell sorting

FasL: Fas ligand

FasR: Fas receptor

FCAS: familial cold-induced autoinflammatory syndrome

fD: factor D

fH: factor H

fI: factor I

FIIND: function to find domain

FOXP3: Forkhead box P3

FSC-A: forward scatter area

G: guanine nucleotide binding

GBP: guanylate binding protein

G-CSF: granulocyte colony stimulating factor

GLUT1: glucose transporter 1

GM-CSF: granulocyte monocyte colony stimulating factor

GPCR: G protein-coupled receptor

GRK: GPCR kinase

GvHD: graft *versus* host disease

HD: healthy donor

HET-E: heterokaryon incompatibility

HIF: hypoxia-inducible factor

HIN: hematopoietic interferon-inducible nuclear protein domain

HIV: human immunodeficiency virus

HMDM: human monocyte-derived macrophages

HMGB1: high mobility group box 1

HSP: heath-shock protein

IBD: inflammatory bowel disease

IC: immune complex

IFI16: IFNγ-inducible protein 16

IFN: interferon

Ig: immunoglobulin

IL: interleukin

IL-1R: interleukin 1 receptor

IL-2R: interleukin 2 receptor

IL-1RA: interleukin 1 receptor antagonist

IL-18BP: interleukin 18 binding protein

IRF: interferon regulatory factor

J: joining

KO: knockout

LAMP1: lysosomal-associated membrane protein 1

LAMTOR5: late endosomal/lysosomal adaptor, MTOR activator 5

LAT1: large neutral amino acid transporter 1

LCMV: lymphocytic choriomeningitis virus

LPS: lipopolysaccharide

LRR: leucine rich repeat

LT: antrax lethal toxin

MAC: membrane attack complex

MAP: MBL-associated proteins

MAPK: mitogen-associated protein kinase

MARCH7: membrane-associated ring finger (C3HC4) 7

MASP: mannose binding lectin-associated serin protease

MBL: mannose binding lectin

MDP: muramyl dipeptide

MDSC: myeloid-derived suppressor cells

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

M-MuLV: Moloney Murine Leukemia Virus

MS: multiple sclerosis

MSU: monosodium urate

mTOR: mechanistic target of rapamycin

MWS: Muckle-Wells syndrome

MyD88: myeloid differentiation gene 88

NACHT: NAIP, C2TA, HET-E and TP1 domain

NAIP: neuronal apoptosis inhibitor protein

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor κ B

NK: natural killer

NKT: natural killer T cell

NLR: Nod-like receptor

NMDA: N-methyl-d-aspartate

NOD: nucleotide binding and oligomerization domain

NOMID: neonatal onset multisystem inflammatory disorder

OXPHOS: oxidative phosphorylation

P2X7: P2X purinoreceptor 7

PAMP: pathogen-associated molecular pattern

PBMC: peripheral blood mononuclear cell

PGE2: prostaglandin E2

PI: propidium iodide

PK: protein kinase

pMHC: peptide-MHC complex

PMN: polimorphonucleated cell

POP: pyrin domain only protein

PRM: pattern recognition molecule

PRR: pattern recognition receptor

PYD: pyrin domain

PYHIN: pyrin and HIN domain-containing protein

RA: reumathoid arthritis

RIG-I: retinoic acid inducible gene 1

RLR: RIG-1 like receptor

ROR-γt : retinoic acid receptor-related orphan receptor gamma-t

ROS: reactive oxygen species

RQ: relative quantification

RT: room temperature

SEM: standard error of the mean

SGT1: suppressor of the G2 allele of skp1

SIRT2: sirtuin 2

SLC2A1: solute carrier family 2 member 1

SLC7A5: solute carrier family 7 member 5

SSC-A: side scatter area

SSC-W: side scatter width

STAT: signal transducer and activator of transcription

T2D: type 2 diabetes

TCC: terminal complement complex

TCR: T cell receptor

Tfh: T follicular helper

Th: T helper

TGF-β: transforming growth factor β

TLR: Toll-like receptor

TNF: tumor necrosis factor

TNFR: tumor necrosis factor receptor

TP1: telomerase-associated protein 1

Treg: T regulatory

TXNIP: thioredoxin-interacting protein

UDA: urticarial deafness amyloidosis syndrome

UNG: Uracil-DNA glycosylase

V: variable

VEGF: vascular endothelial growth factor

WT: wild type

Chapter 1

Introduction

1.1 The immune system

1.1.1 The innate and adaptive immune system: introduction

"An immune system of enormous complexity is present in all vertebrate animals... I find it astonishing that the immune system embodies a degree of complexity which suggests some more or less superficial though striking analogies with human language, and that this cognitive system has evolved and functions without assistance of the brain". This quote by the immunologist Niels Kaj Jerne (The Generative Grammar of the Immune System, Nobel Lecture, 1984) reflects well the complexity and beauty of the mammalian immune system: an intricate network of plasma-soluble factors and different types of cells, which constantly orchestrates a wide range of effector mechanisms for the detection, prosecution, and elimination of exogenous viral, bacterial, or parasitic infections and self-dangerous entities (such as malignant cells) which threaten host viability.

Traditionally, the immune system has been divided into two separate arms, the innate and the adaptive immunity. The former is mostly considered the 'front-line' of host defense, with immunological effectors providing immediate, robust and non-specific responses without the induction of immunological memory. The innate immune system is composed of soluble factors, including the complement system, and specialised cells mostly with phagocytic and secretory functions, such as macrophages, mast cells, dendritic cells (DCs), granulocytes (neutrophils, eosinophils, basophils) and natural killer (NK) cells (Medzhitov and Janeway, 2000; Geering *et al.*, 2013).

The "innate immune" recognition of microbial molecules, named pathogen-associated molecular patterns (PAMPs) and noxious self-derived molecules (derived for example from infected, apoptotic or malignant cells), known as danger-associated molecular patterns (DAMPs), involves different pattern recognition receptors (PRRs) expressed by immune cells. Engagement of these receptors induces several protective effector functions specific for the cell which detects the incoming PAMP/DAMP signal (Medzhitov, 2009). Classic PRRs are the Toll-like receptors

(TLRs), the Nod-like receptors (NLRs) and the retinoic acid inducible gene 1 (RIG-I) like receptors (RLRs), and several proteins of the complement system (reviewed in Creagh and O'Neill, 2006). TLRs belong to a family of membrane-bound receptors which signal via the adaptor myeloid differentiation gene 88 (MyD88), the transcription factors nuclear factor κB (NF-κB) and interferon (IFN)-regulatory factors (IRFs), inducing the expression of pro-inflammatory cytokines such as the interleukins (IL)-6, IL-12, and tumor necrosis factor (TNF)-α (Takaoka *et al.*, 2005). NLRs are cytoplasmic receptors of various microbial and non-microbial stimuli: while activated NOD1 and NOD2 trigger NF-κB signaling and changes in gene expression, other members of this family trigger pro-inflammatory cytokine secretion as part of an inflammasome complex (Guo *et al*., 2015). RLRs are cytoplasmic sensors of RNAs, and their activation leads generally to the induction of type I IFN responses (Loo and Gale, 2011).

The evolutionary younger adaptive immune system, on the other hand, provides selective responses to danger upon identification of specific molecules, known as antigens, by two classes of cell types, B and T lymphocytes. These cells possess an extremely diversified repertoire of antigen-specific recognition receptors which enables the specific detection and clearance of pathogens/danger and the formation of a long-lasting immunological memory, therefore ensuring a rapid and specific response upon re-encounter of the same pathogen (Gourley *et al.*, 2004). While B lymphocytes secrete antibodies (humoral immunity) in the plasma against specific antigens, T lymphocytes provide soluble factors, such as cytokines, in order to enhance immune responses (helper function) or to mediate cell lysis (cytotoxic function) (Luckheeram *et al.*, 2012; Tscharke *et al*., 2015).

1.1.2 T lymphocytes

T lymphocytes are named after their maturation process, from lymphoid hematopoietic stem cell, in the thymus (in opposition to B lymphocytes which mature in the bone marrow), before experiencing further antigen-mediated

differentiation in the peripheral lymphoid organs (Luckheeram *et al.*, 2012). T cell-mediated immunity comprises primary responses by naive T cells, effector functions by activated T lymphocytes, and endurance of antigen-specific memory T cells. T cell-mediated immunity is part of a complex and coordinated immune response that includes other effector innate immune cells. T lymphocytes recognise unique antigenic peptides through the T cell receptor (TCR) expressed on the cell surface; the antigens are presented by antigen-presenting cells (APC) in association with major histocompatibility complex (MHC) molecules (Braciale *et al.*, 1987).

The thymus offers an adequate microenvironment, with a combination of stromal cells, cytokines and chemokines, to generate functional T lymphocytes from cell precursors called "thymocytes". Two critical steps in antigen-specific T cell maturation are the TCR gene rearrangement and the thymocyte selection (reviewed by Starr *et al.*, 2003).

The classic TCR consists of αβ chains (or γδ chains in the sub-class of γδ T cells) associated with different cluster of differentiation 3 (CD3) subunits (the homodimers CD3ζζ and the heterodimers CD3γε and CD3δε). The TCR interacts with the antigen-MHC complex on APCs, while CD3 mediates T cell activation signals (Rudolph *et al.*, 2006). TCR α-chain gene region is composed of V (variable) and J (joining) genes. The β-chain gene region has V, J, and D (diversity) gene segments. A vast repertoire of TCR αβ is generated by gene rearrangement in the variable domains and random insertions/deletions in the junction among gene segments (junctional diversity) (Rock *et al.*, 1994).

During the T cell differentiation process, the contact with peptide-MHC complex (pMHC) on distinct thymic APCs plays a key role in the shaping of the T lymphocytes repertoire for antigen recognition (Klein *et al.*, 2009). Thymocytes expressing TCR with either negligible or very high affinity to pMHC die, while thymocytes with TCR of intermediate affinity to pMHC undergo positive selection and differentiate into mature T lymphocytes. Moreover, self-reactive T cell precursors are eliminated by an apoptotic process known as negative selection, thus avoiding aberrant recognition of self-antigens and development of autoimmune disorders. Immature T cells with TCR of low affinity to self-antigens escape the negative selection and, once migrated to the periphery, they are typically "ignorant"

to self-antigens and develop tolerance following activation.

Following the maturation process, naive T cells migrate to secondary lymphoid organs, including the spleen, lymph nodes, and the mucosa-associated lymphoid tissue, where they continuously scan the environment for antigen recognition (Drayton *et al.*, 2006). Upon antigen encounter, TCR engagement triggers a cascade of intracellular signaling events, resulting in activation of transcription factors, such as the nuclear factor of activated T cells (NFAT), and immune gene expression (Woodrow *et al.*, 1993). TCR activation is further augmented by costimulatory molecules, such as the receptor CD28, expressed on all naive T lymphocytes. The dendritic cells (DCs), expressing the CD80 (B7-1) and CD86 (B7-2) ligands for CD28, are the APCs with enhanced ability to stimulate naive T lymphocytes (de Jong *et al*., 2005). The activated T cells promptly proliferate (clonal expansion), move to the sites of antigen presence, and execute effector functions aimed at antigen elimination. The exact nature of effector differentiation depends on cues from the microenvironment, including the cytokine milieu, antigen concentration, type of APCs and engagement of costimulatory molecules (Tao *et al.*, 1997). Cytokines are initially produced by APCs and innate immune cells, and subsequently by the differentiating lymphocytes. Most effector T lymphocytes disappear after the antigenic agent is eliminated, although a small proportion will survive and form memory T cells, which may reside for years in peripheral tissues and lymphoid organs. Therefore upon antigen re-encounter, memory T cells can perform a much more rapid and potent secondary immune response (Luckheeram *et al.*, 2012).

T lymphocytes with an αβ TCR are categorised into different subsets accordingly to their lineage markers and functionality. Two surface co-receptor molecules, CD4 and CD8, define two separate T cell lineages with different effector functions (Luckheeram *et al.*, 2012). Originally the immature thymocyte is $CD4^+CD8^+$ but undergoes a final maturation into $CD4^+CD8^-$ or $CD4^-CD8^+$ T lymphocyte. $CD4^+$ T cells are activated by antigenic peptides presented by MHC class II molecules (only expressed on "professional" APCs such as DCs, macrophages and B lymphocytes) and produce cytokines that can be directly toxic to the target cells or stimulate other immune cells, therefore they are known as effector T helper (Th) cells. $CD8^+$ T cells recognise antigens in the context of MHC class I molecules (expressed on all

nucleated cells) and perform direct granzyme B and perforin-mediated lysis of infected or malignant cells bearing the antigen, therefore they are so-called effector cytotoxic T lymphocytes (CTL). Other distinct cell subsets expressing the TCR, sharing some features with the innate NK cells and with cytotoxic activity, are the CD1d-reactive natural killer T (NKT) cells, which recognise lipidic molecules in association with CD1d (Bendelac *et al.*, 2007), and the CD1d-independent NKT cells, comprising the $CD8^+$ NKT-like cells (Wang *et al.*, 2015).

1.1.3 $CD4^+$ T cells

$CD4^+$ T lymphocytes execute multiple effector functions, ranging from activation of innate immune cells, B lymphocytes, CTLs, non-immune cells, and suppression of immune reactions. According to their function, $CD4^+$ T cells are subdivided into in classical subsets, including T helper cells Th1, Th2, Th17, Th9, Th22, T follicular helper cells (Tfh), and T regulatory cells either induced (iTreg) or type 1 (Tr1). The lineage differentiation is guided by complex network of cytokines signaling and transcription factors accompanied by epigenetic remodeling (Luckheeram *et al.*, 2012).

Th1 cells participate in protection against intracellular pathogens, but their uncontrolled action is also associated with organ-specific autoimmunity (del Prete, 1992). These cells mainly secrete interferon-γ (IFN-γ) and IL-2. IFN-γ activates mononuclear phagocytes, such as macrophages and microglial cells, thereby enhancing their phagocytic function (Murray *et al.*, 1985). IL-2 acts as a growth factor particularly in CTLs proliferation (Gattinoni *et al.*, 2005; Kim *et al.*, 2006) and promotes the development of $CD8^+$ memory T cells (Williams *et al.*, 2006). Th1 differentiation involves expression of the T-box transcription factor (T-bet), as well as the cytokines IFN-γ and IL-12 (Lugo-Villarino *et al.*, 2003; Trinchieri *et al.*, 2003). IFN-γ and IL-12 induce two other transcription factors participating in Th1 maturation, respectively the signal transducer and activator of transcription 1 (STAT1) and 4 (STAT4) (Greenlund *et al.*, 1995; Thierfelder *et al.*, 1996).

Th2 cells partake in the immune responses to extracellular parasites, such as helminths, but play also a role in the induction of asthma and allergy (del Prete, 1992). The main effector Th2 cytokine is IL-4, which induces the secretion of immunoglobulin E (IgE) by B lymphocytes (Steinke and Borish, 2001), as well as the production of pro-inflammatory mediators, including IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and the expression of adhesion molecules for immune cell migration (Doucet *et al.*, 1998). Th2 lineage differentiation requires the transcription factor IRF4, which activates the *IL4* gene promoter (Rengarajan *et al.*, 2002), and STAT6 (induced by IL-4), which upregulates the expression of the Th2 master regulator GATA-binding protein 3 (GATA3) (Kaplan *et al.*, 1996; Zhu *et al.*, 2006).

Th17 lymphocytes are involved in the defence against extracellular bacteria and fungi, via the activation of mononuclear phagocytes, the recruitment of neutrophils and the induction of epithelial antimicrobial responses. Apart from this protective role, hyperactive Th17 cells also participate in the development of autoimmune diseases (Annunziato *et al.*, 2007). The key effector Th17 cytokines are IL-17A and IL-17F, which stimulate the production of pro-inflammatory cytokines (such as IL-1β, IL-6 and TNF-α) and chemokines (in order to recruit immune cells to the inflamed site) (Kolls and Linden, 2004). Th17 differentiation involves the key transcription factor retinoic acid receptor-related orphan receptor gamma-t (ROR-γt), and the cytokine transforming growth factor β (TGF-β), however only at low concentrations and in the concurrent presence of IL-6 (Ivanov *et al.*, 2006; Veldhoen *et al.*, 2006).

Other distinct subsets of $CD4^+$ T helper lymphocytes are the Th9 cells, secreting larger amount of IL-9 and involved in the pathological process underlying asthma and allergic reactions (Veldhoen *et al.*, 2008; Xing *et al.*, 2011), the Th22 cells, which produce mostly IL-22 and participate in the modulation of epidermal immunity (Eyerich *et al.*, 2009), and the T follicular helper (Tfh) cells, located in the follicular areas of the lymphoid tissue, which are critical to the development of antigen-specific B cell immunity (Breitfeld *et al.*, 2000).

T regulatory cells (Treg) play a fundamental role in maintaining the tolerance to self antigens: after pathogen clearance, they negatively regulate immune responses, thereby avoiding exaggerated inflammation and subsequent detrimental immunopathology in tissues (Sakaguchi *et al.*, 2006; Fujio *et al.*, 2010). The main effector Treg cytokines are IL-10 and TGF-β. IL-10 is a potent immunosuppressive cytokine, with the ability to inhibit pro-inflammatory responses from Th1 and innate immune cells, thus limiting tissue damage (Couper *et al.*, 2008). Indeed, Treg cell transfer is currently considered and clinically assessed as an alternative approach to treat autoimmune diseases and to avoid rejection of transplanted organs (Allan *et al.*, 2008). Moreover, Tregs attenuate the allergic inflammation, as Tregs-derived IL-10 and TGF-β can strongly suppress IgE production (Robinson *et al.*, 2004).

Among the peripheral Treg cells are the induced iTregs, differentiating from antigen-primed naive $CD4^+$ cells in a peculiar cytokine milieu; the iTreg cells are therefore distinct from the thymus-derived natural regulatory T cells (nTregs) (Chen *et al.*, 2003). Forkhead box P3 (FOXP3) is the principal nTreg lineage-specific transcription factor, thus nTregs are identified as $CD4^+CD25^+FOXP3^+$ (Fontenot *et al.*, 2003). TGF-β signaling plays a significant role in both Treg subgroup developments, apart from promoting the antagonistic Th17 cells differentiation: TGF-β alone, at high concentration, can divert lineage differentiation towards iTregs, and induce downstream FOXP3 upon TCR activation (Chen *et al.*, 2003). Finally, distinct from iTregs, the regulatory Tr1 cells are other immunosuppressive IL10-producing $CD4^+$ T cells which do not express FOXP3 constitutively (Groux *et al.*, 1997; Roncarolo *et al.* 2014).

1.1.4 $CD8^+$ T cells

The majority of $CD8^+$ T lymphocytes have cytotoxic activity (hence called CTL) and have a principal role in the elimination of cells infected with intracellular pathogens (mostly viruses) and cancer cells (Tscharke *et al.*, 2015). In analogy to Th1 cells, these CTLs produce large amounts of IFN-γ and TNF-α in the effector state, and their differentiation is dependent on the transcription factor T-bet as well as on the

cytokines IL-12 and IFN-γ (Carter and Murphy, 1999; Glimcher *et al.*, 2004). Following activation, IL-2 and IL-15 are the key cytokines involved in the maintenance of CTLs effector functions and the generation of a $CD8^+$ memory T cells pool (Mitchell *et al.*, 2010; Mathieu *et al.*, 2015).

CTLs are highly efficient antigen-specific effector cells, as a single $CD8^+$ CTL is able to destroy more than one target cell bearing foreign antigens, while effectively sparing "healthy bystander" cells. The CTL-mediated killing of selected cells is initiated by the generation of contacts with the target cell and antigen recognition (the so-called "immunological synapse"), followed by the release of cytotoxic granules by CTLs (Stinchcombe *et al.*, 2001). Unlike naive T cells, the cytotoxic activity of effector CTLs is not dependent on costimulatory signals. Upon TCR activation, the two major cytotoxic pathways deployed by CTL are 1) cell lysis via perforin and granzymes (which is Ca^{2+}-dependent) and 2) apoptosis mediated by Fas ligand (FasL)/Fas receptor (FasR) (Lowin *et al.*, 1994; Esser *et al.*, 1998).
The first pathway requires primarily the release of lytic granules, i.e. secretory lysosomes bearing granzymes, perforin and the proteoglycan serglycin. These lytic granules mature into lysosomes containing the lysosomal-associated membrane protein 1 (LAMP1, also known as CD107a), which is expressed only intracellularly in resting cells but detectable on cell surface of activated CTLs (Sanchez-Ruiz *et al*., 2011). Granzymes are serin-proteases and the most studied member is granzyme B, which mostly induces apoptosis via a caspase-dependent mechanism in the target cells (Adrain *et al.*, 2005). Granzyme B can also directly cleave other substrates which facilitates apoptosis in a caspase-independent fashion, such as the Hop chaperone of the heath-shock proteins (HSP) 70 and 90, relieving these from protecting the cell from apoptosis (Bredemeyer *et al.*, 2004), and the Rho-regulated kinase Rock II, which induce the membrane bleb protusions typical of apoptotic cells (Sebbagh *et al.*, 2005). The intracellular release of granzymes into the target cell requires either the receptor-mediated endocytosis or the direct translocation from endocytic vesicles into the cytosol through plasma membrane pores. Perforin is the pore forming protein involved in this second process, and it has structural similarities to the complement protein C9, also participating in membrane pores assembly (Lichtenheld *et al.*, 1988).

Conversely, Fas-mediated apoptosis, a general process not restricted to CTLs, is triggered by the recognition of FasL by FasR, respectively expressed on the cell surface of CTLs and target cells (Lowin *et al.*, 1994). The FasR, a member of the TNF receptor superfamily, holds an intracellular "death" domain which initiates caspase-dependent apoptosis following FasL binding (Ware *et al.*, 1996).

1.2 The complement system

1.2.1 The complement system: an overview

Among the evolutionary most ancient known mechanisms involved in pathogen recognition is the complement system. Complement's discovery dates back over a century ago, when Paul Ehrlich described a system of serum-circulating proteins that has the ability to "complement" the other immune responses, that are antibody-mediated and cell-mediated (Ehrlich and Morgenroth, 1899). The complement system consists of over 50 blood-circulating and cell-expressed proteins, which play a critical role in the recognition and elimination of pathogens and other dangerous entities, supporting innate immune responses and initiation of inflammatory reactions (Kolev *et al.*, 2014). The complement effector molecules exist largely in a precursor state that is activated rapidly in a cascade-like fashion following recognition of PAMPs and/or DAMPs molecules. Complement can be activated in the blood via three main routes, the classical, lectin and alternative pathways, triggered respectively by immune complexes, microbial surfaces, and fluid-phase C3b-like molecule together with specific C3-cleaving plasma proteases; all these pathways are commonly characterised by the sequential proteolytic cleavage of soluble proteins (proenzymes) by a series of proteases (Figure 1.1) (Ricklin *et al.*, 2010).

The classical pathway is initiated by the C1q molecule within the C1 complex (C1q, $C1r_2$, $C1s_2$), which recognises dangerous PRMs (pattern recognition molecules, including both PAMPs and DAMPs) indirectly via binding to IgG- or IgM-coating antigens or via the C-reactive protein (CRP), or directly interacting with specific PRMs. This recognition induces conformational changes within C1q and subsequent activation of the proteases C1r and then C1s (Ricklin *et al.*, 2010). The lectin pathway is triggered via the recognition of microbial carbohydrates by the mannose binding lectin (MBL), belonging to the collectin family of lectins, or by other molecules of the ficolin family, with subsequent activation of the mannose-binding lectin-associated serine proteases (MASPs) (Garred *et al.*, 2009; Ricklin *et al.*, 2010). C1q, MBL and ficolins can recognise, apart from microbial products, also

danger molecules produced by stressed and dying cells (Korb and Ahearn, 1997; Nauta *et al*, 2003). Both the C1 complex and MASPs can cleave the complement molecules C4 and C2, which associates in the formation of the classical and lectin C3 convertase C4b2a (Ricklin *et al.*, 2010).

The alternative pathway is characterised by tonic low-level C3 hydrolysis to C3(H_2O), exposing binding sites for the protease Factor B (fB), which is then cleaved by Factor D (fD) to generate the alternative C3 convertase C3bBb. Properdin (also known as Factor P) amplifies the alternative pathway activation, as this molecule is able to recognise several PAMPs and DAMPs, to attract fluid-phase C3b to the detected PRMs surfaces and to guide the *de novo* assembly of a C3 convertase complex C3bBbP (Fearon and Austen, 1975).

Both the C3 convertases C4b2a and C3bBb catalyse the proteolysis of C3 into C3a and C3b and the subsequent cleavage of C5, either by the classical/lectin pathway C5 convertase C4b2a3b, or the alternative C5 convertase C3bBb3b, into C5a and C5b. Recently, a fourth route of complement activation, known as extrinsic pathway, has emerged, where C3 and C5 can be cleaved by neutral proteases, such as the coagulation proteins thrombin, plasmin, elastase and plasma kallikrein, indicating a functional cross-talk between the complement and the coagulation systems (Figure 1.1) (Sarma and Ward, 2012).

The main effector molecules of complement activation are the anaphylatoxins C3a and C5a, the opsonin C3b and the C5b component of the membrane attack complex (MAC, also known as terminal complement complex, TCC) (Figure 1.1). The anaphylatoxins C3a, ligand of the receptor C3aR, and C5a, which binds the receptors C5aR1 (CD88) and C5aR2 (C5L2, GPR77), are potent chemo-attractants which activate principally monocyte, granulocyte and mast cell migration (and induce enhanced smooth muscle cells contraction and vasodilatation) and effector functions, including degranulation and cytokines secretion (Heeger and Kemper, 2012). C3b, C4b and C1q are opsonins, which facilitate the phagocytosis of coated microbes and damaged cells (Ricklin *et al*., 2010). The generation of the MAC complex is initiated through C5b association with the molecules C6, C7, C8, and then several molecules of C9, which assembly into a membrane pore-forming complex that mediates the lysis of pathogens and target cells (Ricklin *et al.*, 2010).

Apart from the classic anaphylatoxin receptors, other receptors for the complement activation fragments include CR1 to 4, which mostly bind to the C3b degradation product iC3b, and the C1q receptors (cC1qR, gC1qR, C1qRp); commonly, activation of these receptors is required for normal phagocytosis of complement-opsonised targets by scavenger cells (Ricklin *et al*., 2010).

As particularly C3b, C4b and C5b bind in a non-discriminatory fashion to pathogens and host cells, several soluble and cell-bound regulators control complement activation to prevent unwanted host tissue damage. Examples of complement regulators are: 1) the C1 esterase inhibitor (C1-INH), a serpin family glycoprotein that inhibits different proteases of the classical and lectin pathways; 2) the MBL-associated proteins (MAP), which competes with MASP in binding to MBL (Ricklin *et al.*, 2010); 3) factors that accelerate the decay of convertases, such as surface-bound CD55 (also named "decay accelerating factor", or DAF) or fluid-phase Factor H (fH, of CFH) and C4b-binding protein (C4BP) (Kolev *et al*., 2014); 4) Factor I (fI), a serin protease crucial in the inhibition of all complement pathways, which degrades activated C3b and C4b to generate inactive iC3b and iC4b, and which uses as cofactors fH, C4BP, CR1 and CD46 (known also as membrane cofactor protein, MCP) (Nilsson *et al.*, 2011); 5) carboxypeptidases, enzymes that de-activate the complement products C3a, C4a and C5a via removal of a C-terminal arginine residue, generating the des-arginated complement products C3adesArg, C4adesArg and C5adesArg (Klos *et al.*, 2013); 6) negative regulators of the MAC assembly, such as CD59, vitronectin and clusterin (Ricklin *et al.*, 2010).

The existence of several regulatory mechanisms aligns with the fact that, from an ancient mechanism of innate immune recognition and defence in invertebrates, complement has evolved to an effector system that bridges the innate with the adaptive immune responses in vertebrate species (Sunyer *et al.*, 2003). In fact, although the main source of serum-circulating complement proteins and regulators is the liver, complement molecules are also produced locally and expressed by some non-immune cell types (mostly epithelial cells and astrocytes) and by both innate and adaptive immune cells, including monocytes, macrophages, DCs, granulocytes, NK cells, B and T lymphocytes; therefore, these local complement components are able to modulate the immune responses in the peripheral tissues (Heeger and Kemper, 2012; Kolev *et al.*, 2014).

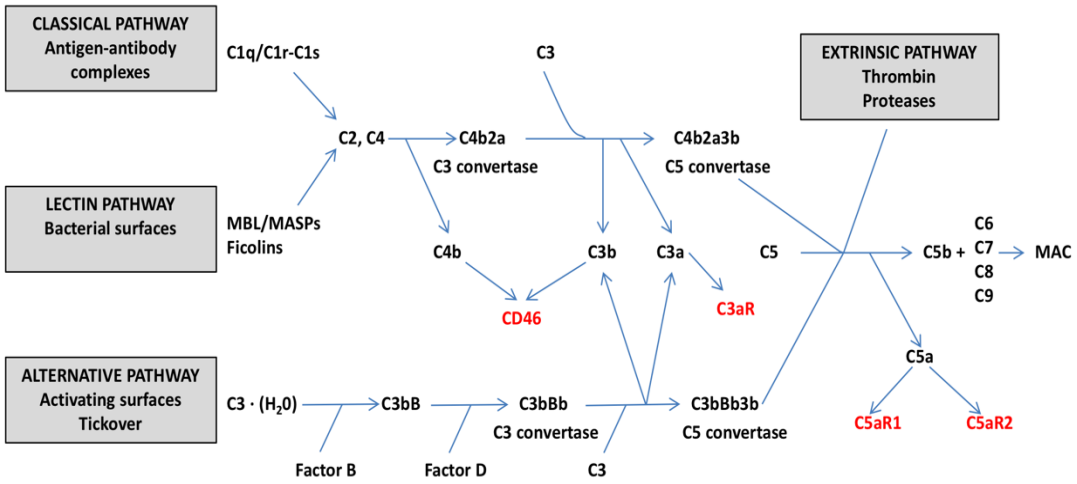


Figure 1.1 The complement cascade
Complement can be activated via three pathways, involving the sequential proteolytic cleavage of soluble proteins by a series of proteases. The classical pathway is triggered by assembly of the proteolytic complex C1 (C1q, $C1r_2$ and $C1s_2$) after binding antigen-antibody immune complexes, while the lectin pathway requires the mannose binding lectin (MBL)-mediated (or ficolins) recognition of microbial carbohydrates followed by activation of the mannose-binding lectin associated serine proteases (MASPs). Both pathways lead to cleavage of C4 and C2 and formation of the C3 convertase C4b2a, that cleaves C3 into the activation fragments C3a (anaphylatoxin) and C3b (opsonin). Tonic, low-level C3 activation occurs in plasma via the 'tick-over' reaction in which C3 is hydrolysed. Plasma circulating $C3(H_2O)$ can bind to the surface of invading pathogenic bacteria. This triggers the binding of Factor B to C3b, and subsequent cleavage of Factor B by Factor D, which generates the C3 convertase C3bBb. Both C3 convertases, after processing additional C3 into C3a and C3b, can bind C3b to form the C5 convertase of the alternative pathway, C3bBb3b, or classical/lectin pathway C4b2a3b. C5 convertase-generated C5a is a potent anaphylatoxin and mediates the inflammatory reaction while C5b binds to C6, C7, C8 and several C9 molecules to form the MAC (membrane attack complex) that mediates pathogen/cell lysis. Finally, activated plasma proteases, such as thrombin, can directly cleave C3 and C5 (extrinsic pathway). Depicted in 'red' are the complement receptors for the terminal complement products, CD46 (receptor for C4b and C3b) and the receptors for the anaphylatoxins C3a (C3aR) and C5a (C5aR1 and C5aR2).
Adapted from Sarma and Ward, 2012, reported in Arbore MPhil upgrade thesis

1.2.2 Complement in adaptive immunity

The best known functions of the complement system are the direct killing of invading pathogens and the mobilization of innate immune cells with the induction of a general inflammatory reaction. It is, however, now increasingly recognised that complement also directly modulates adaptive immune responses, both humoral and cell-mediated (reviewed by Kolev *et al.*, 2014).

The first evidence that complement participates in the regulation of antibody responses dates back to over 40 years ago in studies from Mark Pepys: his work showed that mice depleted of C3 by injection of cobra venom factor (which activates C3 and leads to almost complete C3 'consumption') have diminished T cell-dependent antibody production (Pepys, 1972; Pepys, 1974). A well-known complement mediated mechanism in shaping adaptive humoral immunity is the interaction between the complement receptors CR1 and CR2, expressed by B lymphocytes and follicular DCs in humans and mice, and antigen-bound by the C3 degradation product C3d (Carroll and Isenman, 2012). It has been demonstrated that engagement of CR2, through C3d-coated antigen during B cell receptor (BCR) activation in the lymph nodes, reduces the threshold for BCR signaling and regulates optimal antibody production *in vivo* in mice (Dempsey *et al.*, 1996). Furthermore, follicular B cells in the spleen's subcapsular sinus recognise immune-complexes (ICs) via CR2 and these ICs are captured by macrophages (Phan *et al.*, 2007). Moreover, the follicular DCs bind ICs coated with C3 activation fragments in order to retain the antigens and enhance B-cell memory and effector responses in the germinal centers (Heesters *et al.*, 2013). Via these mechanisms, complement regulates antigen retention and presentation, BCR signaling and ultimately B cell selection, activation and memory formation.

The role of complement in regulating T cell responses is now also well acknowledged, although experimental evidences in support were produced much later when compared to Pepys' studies on humoral immunity. Originally, it was shown by Kopf and collaborators (Kopf *et al.*, 2002) that complement C3-deficient mice have defective T cell priming in the lymph nodes and migration into the lung in

response to pulmonary viral infection, resulting in defective viral clearance. C3-deficient mice have also impaired anti-viral response following systemic infection with lymphocytic choriomeningitis virus (LCMV), the causative agent of meningitis (Suresh *et al*., 2003), associated with diminished antigen-specific $CD8^+$ T cell expansion; conversely, mice deficient for the C3 convertase inhibitor DAF showed enhanced *in vivo* T cell immunity against LCMV, and this effect was reversed by the deletion of C3 or the C5aR1 (Fang *et al.*, 2007). Different complement pathways shape distinct immune responses: in a West Nile virus infection model, it has been shown that mice lacking C4 and FB have reduced anti-viral $CD4^+$ and $CD8^+$ T cell responses and trafficking to the central nervous system (CNS), while no differences were observed among wild type (WT) mice and the C1q-deficient counterpart, thus demonstrating a role specifically for the lectin and alternative complement pathways in regulating anti-viral T cell responses (Mehlhop and Diamond, 2006) – at least in these models. However, C1q binding to human T cells and opsonization of ICs with C1q influences clearly T cell activation and function either directly or via enhanced antigen uptake and presentation by APCs (Chen *et al.*, 1994; van Montfoort *et al.*, 2007), indicating that the roles of complement (activation) fragments/proteins in T cell instruction may depend on the infectious agent and the context of infection.

Studies on the roles of complement in T cell activation led in essence to an important new finding in the complement field. In previous models, complement receptors and regulators were thought to be exclusively activated via complement fragments that had been generated from liver-derived and serum-circulating complement. Work on T cells showed clearly that immune cell-produced 'local' complement activation fragments are critical drivers of T cell activation: locally produced complement anaphylatoxins C3a and C5a control T lymphocytes expansion and differentiation (demonstrated in different *in vivo* disease models of infection and transplantation) either indirectly via modulation of APCs function or via direct stimulation of complement receptors and regulators expressed on T cells (Christmas *et al.*, 2006; Strainic *et al.*, 2008; Lalli *et al*., 2008). Studies in mouse showed that C5a generated by the T cell and/or APC (via secretion of C3 and C5, Factors B and D and subsequent extracellular C3 and C5 convertase formation) binds to the C5aR1 (expressed by $CD4^+$ T cells) in an autocrine fashion and inhibits cell apoptosis thus

enhancing T cell expansion (Lalli *et al.*, 2008). In particular, the autocrine activation of the C3aR and C5aR1 drives the phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB, also known as AKT), mechanistic target of rapamycin (mTOR) and MAP kinases (ERK1/ERK2) pathways in APCs and T lymphocytes, connected with pro-inflammatory Th1 and Th17 response *in vitro* and *in vivo* (Strainic *et al.*, 2008; Sarma and Ward, 2012). Conversely, absence of C3aR and C5aR1 is associated with activation of TGF-β1 signaling and default induction of $FOXP3^+$ iTregs (Strainic *et al.*, 2013). C5aR1 activity also affects DCs function: in the context of TLR2 stimulation, lack of C5aR1 on DCs promotes Th17 and Tregs responses and simultaneously inhibits Th1 induction (Weaver *et al.*, 2010). Local complement anaphylatoxins also modulate Th2 immunity, as demonstrated for both C3a and C5a in models of allergic asthma: for example, C3aR deficient mice showed attenuated airway hyperresponsiveness in a model of pulmonary allergy, associated with reduced Th2 cytokines production in the lung, in line with a positive role for C3a/C3aR signaling in Th2 induction (Drouin *et al.*, 2002); on the other hand, pharmacological targeting of C5aR1 prior to allergen sensitization in a mouse model of allergic asthma resulted in significantly augmented Th2-polarised immune response and allergy inflammation, suggesting a negative role for C5a/C5aR1 signaling in modulating Th2 effector functions (Köhl *et al.*, 2006).

1.2.3 CD46 and C3aR in Th1 induction

Because this thesis work focuses on the role of the intracellular C5 system in human Th1 responses, a detailed introduction is given about the role of complement in the regulation of Th1 immunity, with limited excursions into Th2 and Th17 cells. Before starting this thesis work, the Kemper group had already established that signals mediated by the complement receptors C3aR and CD46, activated in an autocrine fashion by T cell-derived complement activation fragments are critical checkpoints in $CD4^+$ T lymphocytes and Th1 immune responses, controlling the induction, effector and contraction phase (Cardone *et al*., 2010, Le Friec *et al*., 2012, Liszewski *et al*., 2013, Kolev *et al.*, 2014).

The first study indicating a direct role for complement in T cell immunity was

performed by Astier and colleagues and demonstrated that autocrine CD46 signaling induces proliferation and acts as a potent costimulator of human T lymphocytes activation (Astier *et al.*, 2000; Zaffran *et al.*, 2001). CD46 is a transmembrane glycoprotein which is expressed on all human nucleated cells and prevents complement deposition on the host tissues by functioning as cofactor for fI in the cleavage and inactivation of C3b and C4b (Yamamoto *et al.*, 2013). CD46 possesses four distinct isoforms (Figure 1.2), originating from the alternative splicing of a single gene. These isoforms differ in their extracellular *O*-glycolsylation region compositions (termed 'B' and 'C'), and also in the expression of either one of two possible cytoplasmic tails, termed CYT-1 and CYT-2 (Wang *et al.*, 2000; Cardone *et al.*, 2010) – thus, a combination of CD46 B-CYT-1, B-CYT-2, BC-CYT-1 and BC-CYT-2 isoform expression is possible. Both cytoplasmic tails are able to transduce intracellular signals and resting $CD4^+$ lymphocytes mostly express CD46-CYT-2 (Cardone *et al.*, 2010; Le Friec *et al.*, 2012; Liszewski *et al.*, 2013; Kolev *et al.*, 2015).

The critical importance of C3aR and CD46 mediated signals in Th1 induction is exemplified by the fact that patients deficient for C3 or CD46 are unable to mount Th1 responses *in vitro* and *in vivo* and suffer from recurrent infections (Cardone *et al.*, 2010; Cope *et al.*, 2011). Of note, these patients have normal cell proliferation and Th2 responses, demonstrating that C3 and CD46 are specifically required for Th1 induction. Mechanistically, it was shown that CD46 is crucial in up-regulating the expression of CD25 (the IL-2 receptor α-chain) and CD132 (the IL-2 receptor γ-chain), therefore enhancing the ability of T cells to respond to IL-2, a crucial Th1 supporting growth factor (Kolev *et al.*, 2013). Although C3aR signaling during T cell activation is needed for normal Th1 induction (Liszewski *et al.*, 2013), the signals driven by the C3aR during cell stimulation are currently undefined.

Surprisingly, however it turned out that CD46 is also required for the normal function of more basic processes of the cell: before starting the PhD project, and during a MRes rotation in the Kemper laboratory, I collaborated with Dr. Martin Kolev, a postdoc in the lab, on a project aimed at investigating the role of CD46 in human T cell metabolism. This work showed that one of the critical additional functions of CD46 (aside from IL-2R assembly) is to modulate key metabolic events in human $CD4^+$ T lymphocytes, including glycolysis and mitochondrial oxidative

phosphorylation (OXPHOS) (Kolev *et al.*, 2015). Upon TCR stimulation, which induces the autocrine engagement of CD46, CD46 isoforms bearing CYT-1 are upregulated and drive the expression of the glucose and amino acids (AA) channels, specifically the glucose transporter 1 (GLUT1, also known as solute carrier family 2 member 1, gene *SLC2A1*) and the large neutral amino acid transporter 1 (LAT1, alternative name solute carrier family 7 member 5, gene *SLC7A5*), which promotes the nutrient influx necessary for T cell activation. Another gene up-regulated by CD46-CYT-1 is the late endosomal/lysosomal adaptor, MTOR activator 5 (gene *LAMTOR5*), which is involved in mTORC1 assembly, an event crucial for the induction of the high levels of glycolysis (Kolev *et al.*, 2015), specifically needed for IFN-γ secretion and Th1 induction (Chang *et al.*, 2013). CD46-CYT-1 also drives increased OXPHOS in activated T cells and NF-κB signaling (Kolev *et al.*, 2015), and both these events are associated with the metabolic switches required for the induction of T cell effector functions (Mauro *et al.*, 2011; Marelli-Berg *et al.*, 2012). The critical role for CD46 in the metabolic reprogramming of $CD4^+$ T lymphocytes is underpinned by the observation that T cells from CD46-deficient patients have defective glycolysis, OXPHOS, Th1 (and Th17) induction (Kolev *et al.*, 2015).
These data demonstrate not only that autocrine C3-driven signals are needed for normal human Th1 induction but that complement, unexpectedly, participates in the regulation of key metabolic pathways in immune cells.

1.2.4 CD46 in Th1 contraction and homeostasis

Interestingly, C3aR and CD46 mediated signals also contribute to T cell homeostasis and Th1 contraction, respectively. With regards to CD46, this occurs when CD46-mediated signals integrate signals from the IL-2R that respond to increases in high environmental IL-2 generated during the expansion of productive Th1 responses. This CD46-IL-2R crosstalk induces the coexpression of IL-10 in Th1 cells and with that a shift of the effector response toward a (self)regulatory T cell phenotype (Cardone *et al.*, 2010), with the cells finally 'collapsing' into IL-10 single producing T cells. This CD46-driven switch of Th1 cells (from IFN-γ^+ IL-10^- to IFN-γ^+ IL-10^+ and then to IFN-γ^- IL-10^+) is associated with suppression of *IL2* gene expression

(Cardone *et al.*, 2010). IL-10 co-producing Th1 cells themselves proliferate strongly despite their production of this usually anti-proliferative cytokine, but suppress the responses of bystander $CD4^+$, $CD8^+$ and γδ T cells via IL-10 secretion (Barchet *et al.*, 2006). It is proposed that this CD46-driven molecular switch regulates the 'natural life-cycle' of Th1 cells with the purpose of keeping immune responses under tight control and preventing the local over-production of IFN-γ leading to tissue pathologies. This notion is supported by the observation that dysregulation in this molecular switch towards a self-regulatory phenotype has been identified as one strong contributing factor to the hyperactive Th1 response in T cells from patients with reumathoid arthritis (RA) and multiple sclerosis (MS) (Astier *et al.*, 2006; Cardone *et al.*, 2010). The abilities to prevent or to induce this CD46-driven switch in Th1 cells constitute a potential therapeutic mean to stop or reduce chronic infection and cancer or autoimmunity and organ rejection, respectively. However, while there is reasonable understanding about the CD46-driven signaling pathways inducing Th1 responses, much less is known about the CD46 and IL-2R crosstalk that is vital in switching a Th1 cell into the regulatory IL-10 program. The only clue in this regard is the finding that CD46-mediated signals also trigger the switch from high glycolytic activity to steady-state glycolysis in $CD4^+$ cells during IL-10 co-production and Th1 contraction; this change from high to low glycolysis level is mediated by CD46-CYT-2 isoforms, which become the predominant CD46 isoforms in contracting T lymphocytes, similarly to the resting state (Kolev *et al.*, 2015). The mechanism regulating the expression of CD46 isoforms has not yet been defined.
Moreover, there is good evidence that the complement receptors/regulators CR1 and CD59 (expressed on $CD4^+$ T lymphocytes) also negatively control effector T cell responses (Wagner *et al.*, 2006; Longhi *et al.*, 2005). These receptors respectively recognise iC3b and the MAC, generated in sequence after the activating complement fragment C3b and have been shown to both impact negatively on T cell IFN-γ and IL-2 production as well as cell proliferation.

Surprisingly, complement receptors/regulators expressed by $CD4^+$ T cells also modulate T cell homeostasis and this is achieved via a novel crosstalk between complement and the Notch system: on resting T cells, CD46 functions like a 'brake' as CD46 binds the Notch ligand Jagged1 with high affinity and via this sequesters

Jagged1 from an interaction with Notch1 that would usually drive Th1 induction (Le Friec *et al.*, 2012). Upon T cell and autocrine CD46 activation, the downregulation of CD46 on the cell surface allows Jagged1 to bind and activate the Notch1 receptor, and this signaling then drives IFN-γ production. Importantly, T cells isolated from patients with Alagille syndrome, that possess a mutated Jagged1, showed impaired Th1 (but not Th2) cytokines production *in vitro*, in line with the fact that these patients suffer from recurrent ear and respiratory infections (Le Friec *et al.*, 2012).

1.2.5 The concept of intracellular complement activation

Work on the role of complement receptors expressed by T cells in homeostasis of this cell type led to the exciting new discovery of a novel location for complement activation – demonstrating that complement activation is not confined to the extracellular space but can occur within cells. Specifically, human $CD4^+$ T cells contain intracellular stores of C3 and intracellular lysosomal C3aR and cathepsin L (CTSL). CTSL continuously cleaves intracellular C3 into bioactive C3a and C3b in resting cells and engagement of lysosomal C3aR by intracellular C3a sustains T cell survival via low-level mTOR induction. Upon TCR activation, the whole intracellular system translocates rapidly to the cell surface were C3a and C3b signal respectively in autocrine fashion via C3aR and CD46 on the cell surface, triggering IFN-γ production and Th1 induction (Kolev *et al.*, 2013; Liszewski *et al.*, 2013) (Figure 1.3). Thus, the location of complement activation and subsequent receptor engagement (intra- versus extracellular) defines the functional outcome of complement activation. Importantly, the intracellular C3 activation system dictates T cell survival and the magnitude of Th1 responses as 1. T cells in which intracellular C3aR expression is reduced, do not survive (Liszewski *et al.*, 2013) and 2. T cells from patients with idiopathic juvenile arthritis have increased intracellular C3 activation. This hyperactive intracellular C3 system can be normalised by a cell-permeable CTSL inhibitor, which reverses hyperactive IFN-γ production in T cells from these patients to normal levels. Importantly, intracellular C3 activation has been observed in all cells so far analysed (Liszewski *et al.*, 2013) and is therefore

likely of broad physiological significance.

Noteworthy, there are substantial differences among species in the complement-mediated modulation of adaptive immunity. For example, a fully functional murine homologue of CD46 has so far not been characterised, thus there is currently no availability of a suitable mouse model to validate CD46 function better *in vivo*; thus, the role of the CD46 activation is mostly studied in T cells from healthy donors and patients either deficient for CD46 or with Th1-driven autoimmune diseases (Yamamoto *et al.*, 2013). However, a critical importance of both C3aR and C5aR1-mediated signals in Th1 induction has been confirmed in mouse models. For instance, studies using C3aR and C5aR1-deficient animals showed clearly that autocrine $CD4^+$ T cell-derived, but not serum-derived, complement activation fragments deliver the ligands for C3aR and C5aR1, which activate the PI3K/AKT/mTOR pathway in T lymphocytes and drive Th1 effector responses (Strainic *et al.*, 2008; Liszewski *et al.*, 2013; Kolev *et al.*, 2015).

Although the importance of the (intracellular) 'C3 system' to human Th1 biology is now becoming increasingly acknowledged, the possible existence of an intracellular C5 system and its potential role in human $CD4^+$ T cells was unexplored at the beginning of this thesis work.

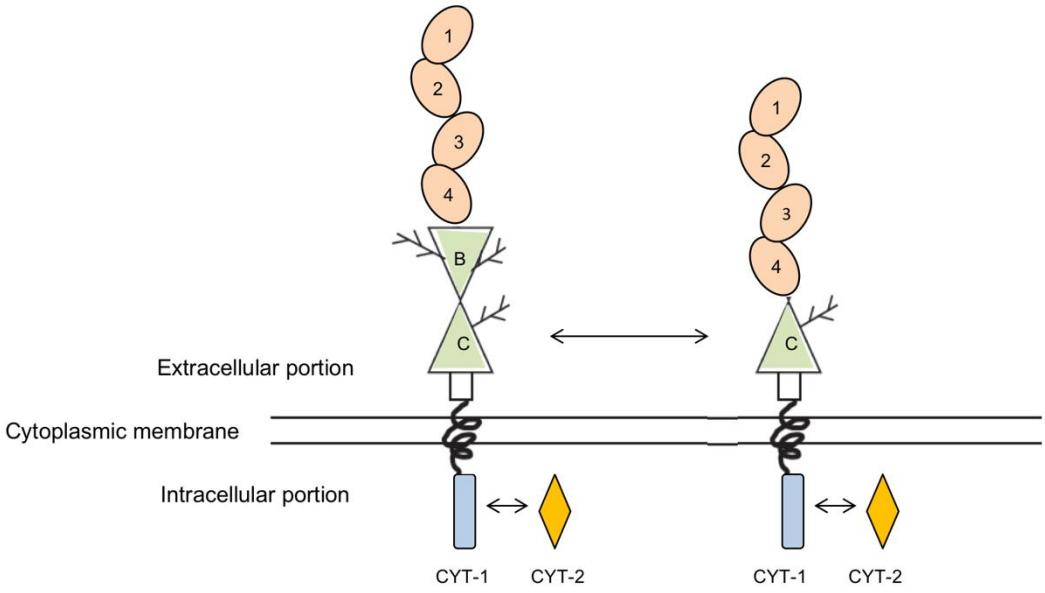


Figure 1.2 Representative scheme illustrating the structure of CD46 and its four isoforms

Alternative splicing of CD46 transcript generates four different isoforms, all composed by four extracellular complement control repeats for ligand binding, a variable *O*-glycosylated region (B and C), a transmembrane portion and one of two different cytoplasmic domains (CYT-1 and CYT-2), both able to signal intracellularly. These four isoforms are designated BC1, BC2, C1 and C2, with the letters referring to the extracellular *O*-glycosylated portion (B and C portions together or C alone) and numbered accordingly to the cytoplasmic tail, CYT-1 or CYT-2.

Extracted from Arbore MRes research report

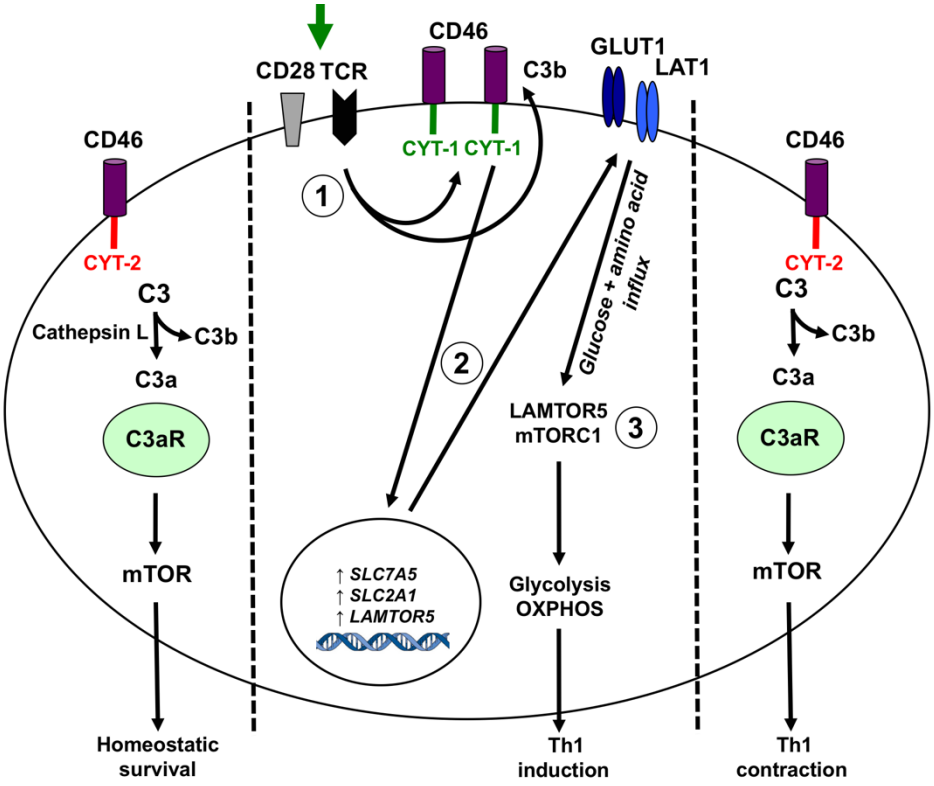


Figure 1.3 Complement in Th1 induction and contraction
C3 activation fragments exert roles in T-cell homeostasis and in the induction of key metabolic events during Th1 responses. In resting T cells, the 'tonic' generation of intracellular C3a via cathepsin L leads to the activation of the C3aR expressed on lysosomes and the low level activation of mTOR which sustains T cell survival (left part of the cell). TCR activation and CD28 costimulation of resting T cells induces the local generation of the CD46 ligand C3b and increased expression of CD46 isoforms bearing CYT-1 (1, middle part of the cell). Autocrine CD46 CYT-1-driven signals then lead to upregulation of genes coding for the glucose transporter GLUT1 (*SLC2A1*), and the amino acid channel LAT1 (*SLC7A5*), allowing for increased influx of glucose and amino acids into the cell (2). In parallel, CD46 CYT-1 mediated signals induce increased expression of *LAMTOR5*, and via this assembly of the lysosome-based machinery enabling amino acid sensing via mTORC1, which then leads the induction of glycolysis and OXPHOS required for IFN-γ production (3). During Th1 contraction and induction of IL-10 co-expression, CD46 isoform expression reverts to a CYT-2 predominant pattern (through a mechanism that is currently unknown) and this is accompanied by reduced expression of GLUT1 and LAT1, down-regulation of glycolysis and OXPHOS and re-instatement of C3a-driven low level mTOR activity (right part of the cell).
From Arbore and Kemper, 2016.

1.2.6 The C5a anaphylatoxin and C5a receptors

The cleavage of the complement protein C5 is towards the end of the complement activation cascade, where the active, C5-derived products C5a and C5b execute a large portion of complement-effector functions (Ricklin *et al.*, 2010). C5 is a 190 kDa protein, composed of an alpha and a beta chain (~ 120 kDa and ~ 75 kDa, respectively) that are connected by disulfide bridges (Sandoval *et al*., 2000). C5 cleavage from the amino-terminus of the alpha chain generates the anaphylatoxin C5a. Human C5a (~ 15 kDa) is composed of 74 AA, forming a globular protein with a four-helix core, stabilised by three disulfide bonds, and a flexible C-terminal 'tail' (AA 64 to 74) (Zuiderweg *et al.*, 1989; Zhang *et al.*, 1997; Huber-Lang *et al.*, 2003). It was long thought that the biological activity and plasma concentration of C5a are controlled by the action of carboxypeptidases, enzymes that catalyse the removal of the C5 carboxyl-terminal arginine residue (Arg), therefore generating the less potent fragment C5adesArg (Bokisch and Müller-Eberhard, 1970; Manthey *et al.*, 2009). Circulating C5a and C5adesArg are quickly cleared by the liver, the renal glomeruli secretion and the internalization after binding to C5a receptors, thus avoiding uncontrolled inflammatory responses due to excessive accumulation of C5a (Manthey *et al.*, 2009). However, since it has now been shown that C5a and C5adesArg binds to both C5a receptors with similar affinity (Croker *et al.*, 2016), it may be possible that des-argination only disactivates C3a.

The C5a anaphylatoxin at nanomolar concentrations is a strong chemoattractant for myeloid cells, such as polymorphonuclear neutrophils (PMNs), basophils, eosinophils, monocytes and macrophages. C5a also induces other pro-inflammatory events, such as smooth muscle contraction, vasodilation, and apoptosis in a range of cells. C5adesArg has similar functions, although higher concentrations are required to trigger biological responses in some cases. Uncontrolled or unwanted C5a generation has been associated with several inflammatory, autoimmune and neurodegenerative disorders (Klos *et al.*, 2009; Woodruff *et al.*, 2011), such as septicaemia (Ward, 2004), autoimmune arthritis (Hashimoto *et al.*, 2010), and MS (Liu *et al.*, 2008). In addition, C5a has been implicated in cancer development, where it promotes angiogenesis, growth factors production, cell proliferation,

invasion and migration, thus preventing anti-tumor immunity (Rutkowski *et al.*, 2010). Furthermore, C5a is not only a potent immunomodulator, but also participates in (re)generative processes, for example, the regulation of embryonic development (Denny *et al.*, 2013; Hawksworth *et al*., 2014).

Aside from instructing innate immune cells, dysregulated modulation of adaptive immune responses by C5a participates in disease development *in vivo*. C5a produced by DCs is required for optimal $CD4^+$ T cell help *in vivo* in allograft rejection (Vieyra *et al.*, 2011), while limiting normal C5aR signaling during DC stimulation induces increased TGF-β, and therefore a drive towards Th17 and Tregs responses (Weaver *et al.*, 2010). As mentioned above, local C5a is required for the survival and effector functions of $CD4^+$ T cells (Strainic *et al.*, 2008). However opposite effects have been ascribed in modulating Th17, with C5a being either a positive or a negative regulator in sepsis (Ma *et al*., 2013) and asthma (Lajoie *et al.*, 2010). In a model of MS, the experimental autoimmune encephalomyelitis (EAE), autoreactive Th1 and Th17 responses have been demonstrated to depend on local generation of C5a (Liu *et al.*, 2008).

While there is only one C3a receptor, two distinct receptors are able to recognise C5a: the C5a receptor 1 (C5aR1 or CD88) and the C5a receptor 2 (C5aR2, alternative names C5L2 and GPR77) (Gerard and Gerard, 1991; Cain *et al*., 2002). These C5a receptors belong to the G protein-coupled receptors (GPCR) and seven transmembrane domains receptor superfamily (Joost and Methner, 2002): they possess an extracellular N-terminus, seven transmembrane domains connected by intra- and extra-cellular loops, and a cytoplasmic C-terminus (Findlay and Pappin, 1986). C5aR1 and C5aR2 are known to have important functions in many inflammatory conditions (Monk *et al.*, 2007). While initial studies found that C5a binds both receptors with the same affinity (Kd ~2.5 nm) and C5aR2 recognises C5adesArg with higher affinity (Kd ~12 nm) than C5aR1 (Kd ~660 nm) (Higginbottom et al., 2005), recent work suggests that both receptors bind C5a and C5adesArg with similar affinities (Reis *et al.*, 2012; Croker *et al.*, 2014) and the reasons for these discrepancies are currently unclear. C5aR1 and C5aR2 are expressed by different immune and non-immune cells, including macrophages,

granulocytes, lymphocytes, adipocytes, skin fibroblasts, neurons and astroglia of the central nervous system (CNS), and in different tissues, such as liver, kidney, lung, heart, ovary, testis, thyroid, adrenal gland and spinal cord (Klos *et al*., 2009; Li *et al*., 2013; Klos *et al*., 2013). However, expression by lymphocytes remains controversial, with some reports showing the expression of these receptors on B and T cells (reviewed in Klos et al., 2009) and some not (Dunkelberger *et al*., 2012). Another matter of debate is the cellular localization of the C5aR1 and C5aR2 (Sarma and Ward, 2012): while studies on PMN cells detected only intracellular C5aR2 and both cytoplasmic and membrane surface expressed C5aR1 (Bamberg *et al*., 2010), the expression pattern of these receptors on human T lymphocytes and other immune cells has not been clearly defined.

1.2.7 The C5a receptor 1

The human C5aR1 was cloned 25 years ago (Boulay *et al.*, 1991; Gerard and Gerard, 1991) and it has a mass of ~42 kDa with 350 AA. In analogy to the other GPCR, ligand binding leads to a conformational change in C5aR1 and the downstream activation of guanine nucleotide-binding proteins (G proteins). G proteins are composed by three subunits, α, β, and γ, and their activation leads to the following events: 1) guanosine diphosphate (GDP) (bound to the Gα subunit) is exchanged for guanosine triphosphate (GTP); 2) Gβ and Gγ subunits dissociate from Gα, therefore intracellular signals are triggered by molecules such as cyclic adenosine mono-phosphate (cAMP) and calcium mitogen-activated protein kinases (MAPK); 3) signaling is terminated via phosphorylation of the GPCR cytoplasmic tail by GPCR kinases (GRKs) and/or recruitment of β-arrestin (Rosenbaum *et al*., 2009). β-arrestin modulates receptor signaling and trafficking (Gurevich and Gurevich, 2006), in particular by inhibiting G protein binding (and possibly also by triggering G protein independent signals) therefore promoting receptor desensitisation and internalisation through clathrin-coated pits (Rajagopal *et al.,* 2010). It seems that, depending on the agonist and the resultant conformational changes in GPCR, either the G protein or the β-arrestin pathways are preferentially activated (Rajagopal *et al.*, 2010). Activated C5aR1 has been shown to associate with the β-arrestins 1 and 2 (Braun *et*

al., 2003), which modulates receptor signaling. Furthermore, the C5aR1-induced signaling ability can be inhibited by intracellular proteases: purified neutrophil serin proteases, neutrophil elastase, cathepsin G and proteinase 3 cleave C5aR1 to a 26 to 27 kDa membrane-bound fragment, thereby inactivating this receptor (van den Berg *et al.*, 2014).

The binding of C5a to C5aR1 triggers several signaling pathways, including PI-3K/AKT (Wrann *et al.*, 2007), phospholipase D (Mullmann *et al.*, 1990), protein kinase C (PKC) and MAPK pathways (la Sala *et al*., 2005). C5aR1 signaling stimulates: 1) the recruitment of immune cells; 4) the production of cytokines and chemokines by immune cells; 3) the expression of cell adhesion molecules by endothelial and epithelial cells, and the vasodilatation; 4) the chemotaxis, phagocytosis and degranulation by neutrophils (Guo and Ward, 2005).
Another mechanism, aside from internalisation and desensitation, which modulates C5aR1 function is the formation of receptor oligomeric structures: Klco *et al*. (2003) demonstrated the cross-linking of C5a receptors in membranes prepared from both human neutrophils and stably transfected Chinese hamster ovary (CHO) cells; the formation of oligomers may be linked to C5aR1 cross-modulation or rapid down-modulation, such as in the severe condition of sepsis. Additional studies further demonstrated the ability of the C5aR1 to form homodimers and heterodimers (Geva *et al*., 2000; Hüttenrauch *et al.*, 2005; Klco *et al.*, 2003), where ligation of a dimerised C5aR1 can lead to phosphorylation and/or internalization of the partner receptor. Particular attention has been given to the ability of C5aRs heterodimers formation since it has been discovered that C5aR1 can form heterodimers with the C-C chemokine receptor 5 (CCR5), a receptor that facilitates the entry of the human immunodeficiency virus (HIV) into the host cells (Hüttenrauch *et al.*, 2005). C5aR1 can also form oligomers with the alternative C5a receptor, C5aR2, which then negatively regulates C5aR1 signaling (Croker *et al.*, 2013).

1.2.8 The C5a receptor 2

The alternative C5a, receptor, C5aR2, was cloned by Ohno *et al*. (2000), with a predicted molecular weight of 37 kDa and length of 337 AA. The C5aR2 is also a seven transmembrane receptor and it shares a 35% sequence homology with C5aR1 (Figure 1.4) (Woodruff *et al.*, 2011). C5aR2 (similarly to C5aR1) contains acidic and tyrosine residues in the N-terminal region and hydrophobic and charged residues in the transmembrane portion that facilitate binding of C5a (Monk *et al.*, 2007). As mentioned before, C5aR2 can under certain circumstances bind C5adesArg with higher affinity than C5aR1, most likely due to specific residues at the N-terminus (Scola *et al.*, 2007). Further, the intracellular loop 3 of C5aR2 lacks a basic Lys-Thr-Leu-Lys (K-T-L-K) motif, which is present in C5aR1 and required for signal transduction; indeed, no calcium mobilization and MAPK pathway activation have been observed in cells stably transfected with C5aR2 upon C5a stimulation (Okinaga *et al.*, 2003). Finally, and differently from C5aR1, C5aR2 appears to be uncoupled from G proteins, as it lacks the Asp-Arg-Tyr (D-R-Y) motif, which in C5aR1 is Asp-Arg-Phe (D-R-F) and in C5aR2 is Asp-Leu-Cys (D-L-C), and the Asn-Pro-X-X-Tyr (N-P-X-X-Y) motif in the transmembrane helix 7, both motifs required for G protein binding (Okinaga *et al.*, 2003). Apart from recognising C5a and C5adesArg, C5aR2 has also been suggested to act as receptor for C3adesArg, known also as acylation-stimulating protein (ASP), which is involved in lipid and carbohydrate metabolism (Paglialunga *et al.*, 2007). However there is some data controversy among different research groups (Johswich and Klos, 2007) and the prevailing opinion is currently that C5aR2 cannot bind C3adesArg.

In most cells and tissues, C5aR2 is co-expressed with C5aR1 and generally at a level lower than C5aR1 (Okinaga *et al.*, 2003; Lee *et al.*, 2008; Bamberg *et al.*, 2010). The exact C5aR2 expression profile, specifically in human myeloid cells, has been a matter of discussion for a long time, and it is now suggested that there is a wide variation in C5aR2 surface expression among human cells, possibly due to the cell activation status and the cell type, where C5aR2 expression level may modulate cellular functions and immune responses (Scola *et al.*, 2009).

Although the C5aR1 is definitely categorised as ‘pro-inflammatory’ receptor, the

role of C5aR2 is still enigmatic, with three main different function hypothesised (Figure 1.5): 1) C5aR2 may act as non-signaling decoy receptor, which antagonises the pro-inflammatory signals usually triggered by the C5aR1 (Scola *et al.*, 2009); 2) C5aR2 can bind to β-arrestins, by which it modulates C5aR1 signaling (Van Lith *et al* 2009; Bamberg *et al.*, 2010); 3) C5aR2 can independently and actively transduce either pro- or anti-inflammatory signals, with effects varying in respect to the cell type and disease setting (Bamberg *et al.*, 2010; Li *et al.*, 2013).

The "decoy" hypothesis suggests that C5aR2 may compete with the C5aR1 for C5a and C5adesArg bioavailability by sequestering these ligands from the extracellular environment and targeting them for intracellular degradation (Figure 1.5, panel A) (Okinaga *et al.*, 2003; Scola *et al.*, 2009). This hypothesis is corroborated by the observation that C5aR2 is uncoupled from G proteins and that C5aR2 ligands are constitutively internalised (via a clathrin-dependent mechanism) and degraded in human PMN cells (Klos *et al.*, 2009; Scola *et al.*, 2009; Li *et al.*, 2013). Several groups reported that *in vitro* binding of C5a, C3a, C4a, or their desArg derivatives to C5aR2 does not trigger G protein-mediated responses such as degranulation, chemotaxis, Ca^{2+} mobilization and MAPK pathway activation, in contrast to C5aR1 activation (Cain and Monk, 2002; Kalant *et al.*, 2003; Okinaga *et al.*, 2003; Johswich *et al.*, 2006; Scola *et al.*, 2009). On the basis of these observations, it has been proposed that C5aR2 plays a fundamental role in removing the more abundant C5adesArg form from circulation, as ligand internalisation seems more efficient for C5adesArg than for C5a (Scola *et al.*, 2009).

An emerging alternative idea is that C5aR2 engages G protein-independent signaling, such as β-arrestin mediated pathways (Figure 1.5, panel B) (Defea, 2008): C5a-induced translocation of β-arrestin 2 has been observed in cells stably transfected with C5aR2, where upon C5a binding, the C5aR2 physically interacts with both β-arrestin and the C5aR1 to prevent C5aR1-mediated signals, in particular ERK1/2 phosphorylation (Cui *et al.*, 2009; Van Lith *et al.*, 2009; Bamberg *et al.*, 2010). Specifically, in human monocyte-derived macrophages (HMDM), C5a, but not C5adesArg, induces heterodimer formation of C5aR1 and C5aR2 (Croker *et al.*, 2013), where C5aR2 recruits β−arrestin and down-modulates the ERK1/2 signaling triggered by C5aR1 (Croker *et al.*, 2014).

Finally, in line with the third hypothesis that C5aR2 acts as active signals transducer

(Figure 1.5, panel C), it has been shown that *in vitro* stimulation using C5a leads to diminished activation of MAPK pathways in neutrophils and macrophages from C5aR2$^{-/-}$ animals (Chen *et al.*, 2007), and that, during sepsis, C5aR2 stimulates the release of high mobility group 1 box proteins (HMGB1) (Rittirsch *et al.*, 2008), the latter playing an important role in the pathogenesis of autoimmune arthritis (Andersson and Harris, 2010).

To make the situation even more complex, aside from differences in the signaling pathways induced, both anti- and pro-inflammatory effects have been ascribed to C5aR2 *in vivo* in disease models. Both the decoy and β-arrestin mediated functions are in line with an anti-inflammatory role for C5aR2, where C5aR2 negatively regulates C5aR1-mediated responses (Scola *et al.*, 2009). Congruent with this, mice deficient for the C5aR2 mounted an exaggerated inflammatory reaction, with increased neutrophils influx into the lung and higher levels of IL-6 and TNF-α in a model of ovalbumin (OVA)-induced pulmonary injury (Gerard *et al.*, 2005). Further, increased C5aR2 expression in rat astrocytes is associated with suppression of pro-inflammatory genes (Gavrilyuk *et al.*, 2005). In a rat model of sepsis-induced by cecal ligation and puncture (CLP), C5aR2 protein expression is increased in the liver and lung, and blocking C5aR2 via specific antibodies leads to elevated IL-6 levels in serum, while rat neutrophils pretreated with a C5aR2-blocking antibody produced higher amounts of IL-6 compared to untreated cells in presence of C5a and lipopolysaccharide (LPS) (Gao *et al.*, 2005). Similarly, a study of human septic patients reported that low C5aR2 expression on PMN cells correlated with sepsis-induced multi-organ failure and reduced survival, and similar results were observed in rats subjected to CLP-induced sepsis (Huber-Lang *et al.*, 2005). In support for an anti-inflammatory role in adaptive immunity, C5aR2 was found to be upregulated by TGF-β, in turn increasing the induction of anti-inflammatory iTregs (Strainic *et al.*, 2013).

In contrast to these experimental evidences, C5aR2 was also shown to support C5aR1 activity in neutrophils, macrophages and fibroblasts, therefore having a pro-inflammatory role in models of sepsis (Gao *et al.*, 2005; Chen *et al.*, 2007; Rittirsch *et al.*, 2008) and allergic asthma (Zhang *et al.*, 2010). Specifically, C5aR2-deficient mice showed reduced OVA-induced airway hyper-responsiveness and inflammatory

cell infiltration, although these mice were more susceptible to LPS-induced shock, associated with elevated IL-1β serum levels and high mortality (Chen *et al.*, 2007). The same group has also reported attenuated immune complex-induced lung injury in C5aR2$^{-/-}$ mice, in contrast with the study from Gerard and collaborators (Gerard *et al.*, 2005). Regarding a function in sepsis, it seems that only a combined pretreatment with both anti-C5aR1 and anti-C5aR2 antibodies reduce lethality, with C5aR2 causing sepsis via release of HMGB1 (Rittirsch *et al.*, 2008). Further, the C5aR2 plays a complex role in the pathogenesis of experimental allergic asthma, also in regulating adaptive immune responses: C5aR2 acts at the DC/T cell interface to suppress Th1 and Th17 polarization and concomitantly drives Th2 cytokine production (Zhang *et al.*, 2010).

In conclusion, although it is now broadly acknowledged that C5aR2 activation participates in key immunological processes (Li *et al.*, 2013) the exact physiological roles of the C5aR2 under both normal and pathological conditions remain a matter of debate and likely depend on the cell type that expresses this receptor and the context in which the cell is activated.

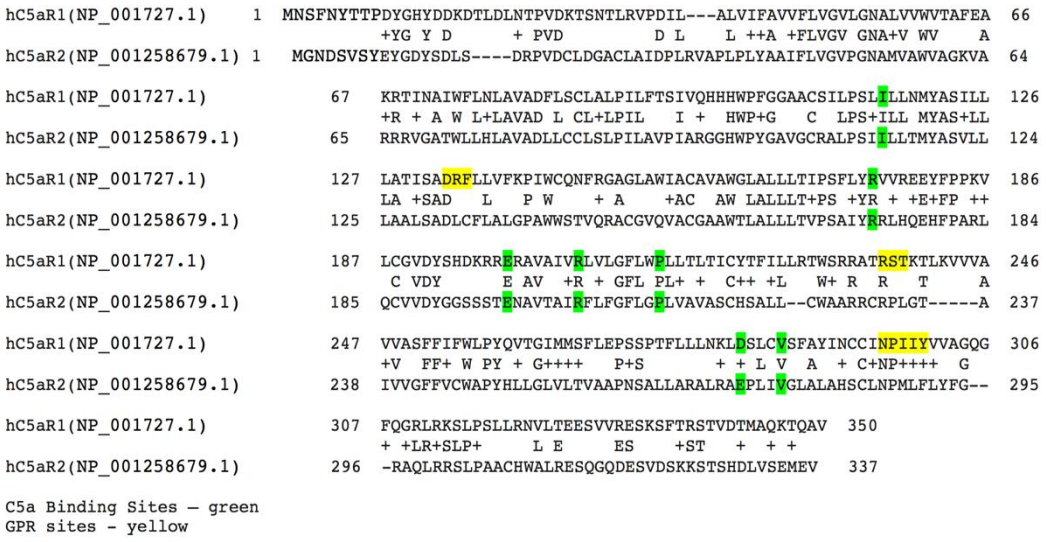


Figure 1.4 C5aR1 and C5aR2 structure
P-blast alignment of the FASTA protein sequences for human C5aR1 and C5aR2 (NCBI website), with the conserved C5a binding sites green (extracted from Klos *et al*., 2013) highlighted in and G protein-coupled receptor (GPR) binding sites (reported by Monk *et al*, 2007) indicated in yellow.

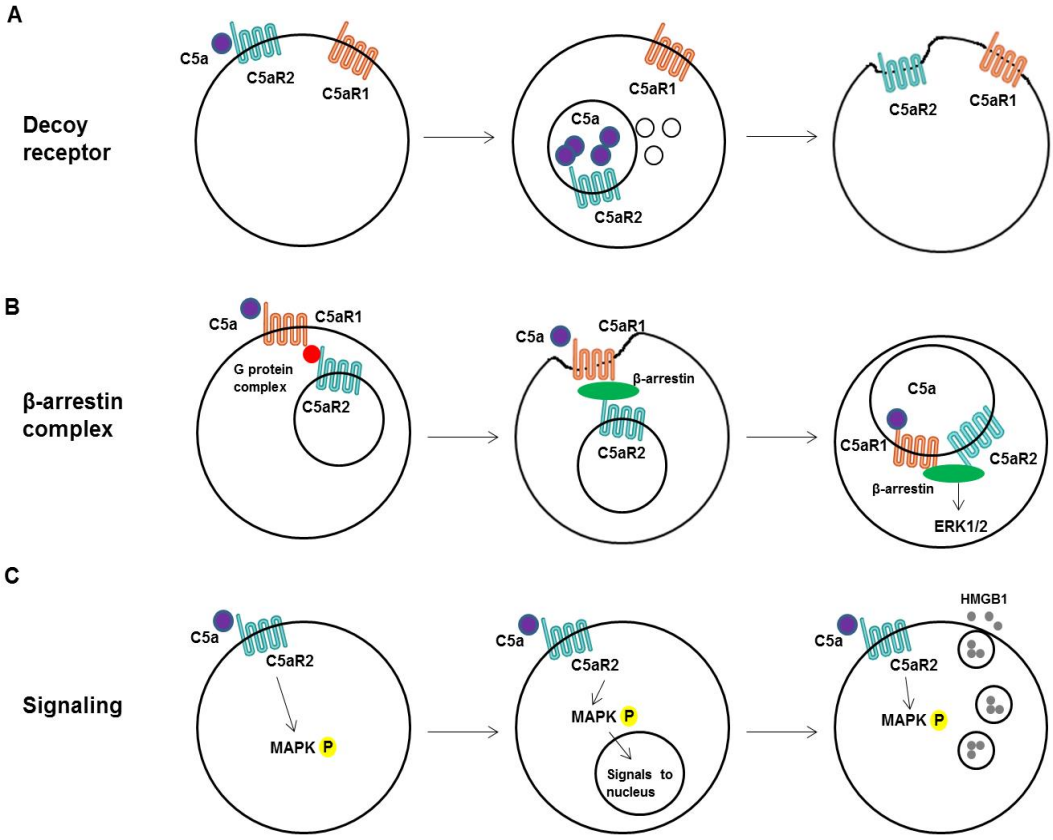


Figure 1.5 Models for possible C5aR1/C5aR2 interactions and cross-regulations
The biological role of C5aR2 is not fully explored yet and a matter of controversy in the field. Shown are the three current models for its potential function. (A) The decoy receptor model states that C5aR2 on the cell surface prevents C5a (generated locally or in serum) from binding C5aR1 and thereby inhibits the normally pro-inflammatory role of C5aR1-driven signals. (B) In the 'β-arrestin-coupling' model, C5aR2 is only expressed intracellularly. When surface C5aR1 binds C5a, this complex is internalised and pairs then with the C5aR2 receptor. This complex recruits β-arrestin, which prevents the usual induction of ERK1/2 phosphorylation driven by C5aR1 activation. In the model shown in (C), C5aR2 has an active signaling function independent of C5aR1-mediated signals. C5aR2 activation has been demonstrated to stimulate the release of HMGB1 (high mobility group box 1) protein through MAP kinases phosphorylation and activation.
Adapted from Li *et al*., 2013, figure extracted from Arbore PhD research proposal

1.2.9 Therapeutic targeting of the C5a system

Modulation of the C5a/C5a receptors axis is crucial for elimination of pathogens/dangers and equally important in cell homeostasis and disease prevention. However, unwanted or uncontrolled C5a generation has been linked to several pathologies, including asthma, sepsis, acute lung injury, ischemia-reperfusion injury, atherosclerosis, glomerulonephritis, dementia, MS and RA. Thus, approaches targeting C5a/C5aRs signaling have been at the center of interest for complement-targeted therapeutic intervention (Sarma and Ward, 2012).

A valuable therapeutic strategy is the direct targeting of C5a generation: Eculizumab (also known as Soliris) is a humanised monoclonal antibody, developed by Alexion Pharmaceuticals (Cheshire, CT), that binds to C5 and prevents generation of C5a and C5b and with this also the assembly of the MAC (Thomas *et al.*, 1996). Eculizumab is currently approved for the treatment of paroxysmal nocturnal hemoglobinuria, a disease characterised by frequent red blood cell lysis, severe anemia and venous thrombosis (Hill *et al.*, 2005; Woodruff *et al.*, 2011).

On the other hand, efforts in targeting the C5aR1 have been concentrated on anti-C5aR1 antibodies, peptides, and small molecule inhibitors (Monk *et al.*, 2007). A selective potent C5aR1 antagonist, with no measurable effects on C5aR2, is PMX53 (or 3D53), a cyclic hexapeptide derived from the C-terminus of C5a, AcF-[OPdChaWR], with pIC_{50} = 7.05 assessed on human neutrophil membranes (Strachan *et al*., 2000, Scola *et al*., 2007). PMX53 is currently produced by Teva Pharmaceutical Industries Ltd (Petah Tikva, Israel) and has completed clinical Phase II trials for treatment of psoriasis and RA, unfortunately, without demonstrating substantial beneficial effects (Woodruff *et al.*, 2011). In addition, pharmacokinetics characteristics limit the therapeutic usage of PMX53, because this compound is characterised by a short half-life and is rapidly degraded in the intestinal mucosa (Woodruff *et al.*, 2005; Morgan *et al.*, 2008).

Based on the numbers of studies reporting an anti-inflammatory role for C5aR2, in preventing C5aR1 pro-inflammatory signaling, treatments that increase C5aR2 expression or activity could be used as anti-inflammatory therapeutic intervention (Monk *et al.*, 2007). Noteworthy, there is lack of selective ligands for C5aR2,

therefore C5aR2-specific activity has until now been studied *in vitro* and *in vivo* by using C5aR2 deficient animals, C5aR2 knockdown or blocking antibodies (Croker *et al.*, 2016). While several blocking antibodies targeting the mouse, rat, or human C5aR2 have been developed and tested *in vitro* and *in vivo* (Gao *et al.*, 2005; Cui e*t al.*, 2007; Lee *et al.*, 2008; Bamberg *et al.*, 2010), the only published antagonists for the human C5aR2 is the large molecule C5a antagonist $A8^{\Delta 71-73}$, which does inhibit ligand binding to both the C5aR1 and C5aR2, and therefore it is a double receptor antagonist (dRA) (Heller *et al.*, 1999; Otto *et al.*, 2004). However, very recently, functional selective C5aR2 ligands have been described, which can recruit β-arrestin 2, inhibit IL-6 release and C5a-induced neutrophil mobilization *in vivo*, and modulate C5a-induced ERK1/2 activation in HMDMs (Croker *et al.*, 2016). These two peptides (P32, Ac-RHYPYWR-OH, and P59, Ac-LIRLWR-OH) at 100μM concentration showed significant inhibition of ^{125I}C5a binding to C5aR2 in radioactive binding studies but do not interact with the C5aR1. These ligands provide an effective tool for complement researchers to selectively probe C5aR2 function and deliver a medicinal chemistry template for development of higher selective and more potent C5aR2 ligands - that may have potential as treatments for inflammatory and other complement-mediated diseases (Croker *et al.*, 2016).

1.3 The NLRP3 inflammasome

1.3.1 A brief introduction to inflammasomes

The execution of the immune system effector functions requires initially appropriate PAMPs and DAMPs recognition by PRRs expressed by immune cells. Among the key effector functions triggered by PRRs, in particular NLRs, is the induction of the assembly and activation of a macromolecular protein complex called "inflammasome". This complex triggers the maturation and production of the key host pro-inflammatory cytokines IL-1β and IL-18, fundamental in regulating pathogen and danger clearance (Dowling and O'Neill, 2012). Recently, experimental evidences demonstrate a role for the complement system (aside from the TLRs as 'classic' inflammasome inducers), particularly engagement of complement receptors and regulators on immune cells, either independently or in conjunction with incoming signals from other PRRs, in the activation and regulation of inflammasome function (Asgari *et al.*, 2013; Samstad *et al.*, 2014).

1.3.2 The inflammasome: complex regulating IL-1β and IL-18 secretion

The inflammasome was first discovered and defined as a functional multiprotein complex in the last decade by Martinon (Tschopp *et al.*, 2003). As mentioned above, this innate immune sensor regulates the production of the mature pro-inflammatory cytokines IL-1β and IL-18, both belonging to the IL-1 cytokine family. These cytokines are synthesised as the inactive precursors pro-IL-1β (31 kDa) and pro-IL-18 (24 kDa) which require proteolytic cleavage mediated by caspases (cysteine-dependent aspartate-directed proteases), mostly caspase-1, to become mature and bioactive IL-1β and IL-18 (both 17 kDa) (Dinarello, 2005; Dinarello *et al.*, 2013). Caspase-1 also exists as an inactive preform in cells and it is proteolytically activated by the inflammasome, therefore functional caspase-1 cleaves pro-IL-1β and pro-IL-

18, and the active cytokines are then secreted outside of the cell. IL-1β and IL-18 are mostly produced by myeloid cells, such as monocytes, macrophages and neutrophils, and facilitate their effector functions during the clearance of pathogens and dying or damaged self cell (van de Veerdonk *et al.*, 2011).

IL-1β can bind to two receptors expressed on immune cells, the IL-1 receptor 1 (IL-1R1), whose signal transduction is mediated by the MyD88 adaptor protein, and the IL-1 receptor 2 (IL-1R2), that has currently no defined signal transduction function (Arend *et al.*, 2008). Other endogenous immune and non-immune cells derived ligands for IL-1 receptors are the pro-inflammatory IL-1α cytokine, which is usually not activated by inflammasomes, and the natural IL-1 receptor antagonist (IL1RA), which does not possess agonistic activity and therefore acts as an antagonist for the binding of IL-1α and IL-1β to the IL-1Rs (Dinarello, 1996). IL-1 receptor 1 signals via several intracellular adaptor molecules but principally through MyD88, and activates different signal transduction pathways, such as NF-κB, AP-1 (activator protein-1) and p38 MAPK pathways (Arend *et al.*, 2008). Interleukin-1β exerts many fundamental function in innate immunity (Sims and Smith, 2010, Di Virgilio, 2013), such as: 1) stimulation of immune cells to release other pro-inflammatory cytokines, including TNF-α, IL-6, IL-1α and IL-1β itself, 2) induction of release of growth and differentiation factors crucial for immune cells, like GM-CSF and granulocyte colony-stimulating factor (G-CSF), 3) secretion of vascular endothelial growth factor (VEGF) and expression of adhesion molecules on vascular endothelial cells, fundamental for cell migration during inflammation, and 4) DCs activation. Moreover, IL-1β participates in adaptive immunity and lymphocytes activation, via modulation of T cell priming and T helper effector function (Zielinski *et al.*, 2012), specifically supporting the differentiation into Th1 and Th17 effector cells (Rao *et al.*, 2007) and the generation of memory T cells (Schenten *et al.*, 2014).

The ‘other’ IL-1 family cytokine that needs to be processed by the inflammasome to become fully active is IL-18. This cytokine was originally defined as IFN-γ inducing factor, for its ability to stimulate IFN-γ secretion in the serum of mice after intraperitoneal injection of endotoxin (Nakamura *et al.*, 1989). The IL-18 receptor (IL-18R) has a structure similar to IL-1R1, recruits the same adaptor molecules and activates the same signal transduction pathways. Immune and non-immune cells can

also secrete a soluble form of the IL-18 receptor, the IL-18 binding protein (IL-18BP), which sequesters IL-18 therefore inhibiting IL-18 binding to the IL-18 receptor (Arend *et al.*, 2008). Similar to IL-1β, IL-18 has many functions in the regulation of both innate and adaptive immune responses such as: 1) enhancement of functional cytokines secretion by NK cells, basophils and mast cells, 2) induction of IFN-γ production by T lymphocytes, and 3) sustainance of Th1 and CTLs function (Okamura *et al.*, 1995; Smith, 2011; Dinarello *et al*. 2013).
Considering the broad range of effector functions triggered by IL-1β and IL-18, it is not surprising that dysregulated secretion of these cytokines is a key pathological feature of chronic inflammatory, metabolic and autoimmune diseases.

Different inflammasomes, once activated, can assemble and drive IL-1β and IL-18 maturation. The inflammasome is a multiprotein complex, composed of PAMPs/DAMPs sensor molecules, either of the Nod-like receptor (NLR) or the PYHIN (pyrin and HIN domain-containing protein) family, caspase-1 and eventually adaptor molecules for the interaction among the sensor protein and the caspase (Figure 1.6) (Dowling and O'Neill, 2012).
Over twenty human NLRs have been identified on the basis of their molecular structure and phylogeny (Ting *et al.*, 2008; Schroder and Tschopp, 2010), and the currently best characterised are NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, all composed of the NOD (nucleotide binding and oligomerization domain), LRR (leucine rich repeat) and pyrin domains, and NLRC4 (known also as IPAF), containing the domains NOD, LRR and CARD (caspase recruitment domain) (Figure 1.6). The NLR proteins contain three main domains: 1) an amino-terminal death-fold domain involved in the formation of the multiprotein complex, where NLRPs contain a pyrin domain, which binds indirectly to caspase-1 through the adaptor ASC (apoptosis speck protein, also possessing a pyrin domain), while NLRC4 contains a CARD, that can directly bind caspase-1 without need for an adaptor molecule; 2) a central NACHT (NAIP (neuronal apoptosis inhibitor protein), C2TA (class 2 transcription activator, of the MHC), HET-E (heterokaryon incompatibility) and TP1 (telomerase-associated protein 1)) domain, involved in nucleotide-binding and oligomerization; 3) carboxy-terminal repeat LRRs that most likely have a role in regulating ligand binding (Ting *et al.*, 2008). An exception to

the NLRPs proteins is represented through NLRP1, which has two additional domains at the C-termini, a FIIND (function-to-find) domain involved in autolytic proteolysis (Finger *et al.*, 2012) and a CARD domain. Both NLRP1 and NLRC4 can interact and activate caspase-1 through their CARD domain without recruiting the adaptor ASC; despite this, the recruitment of ASC in the case of NLRP1 strongly enhances the formation of a functional inflammasome complex (Proell *et al.*, 2013).

The first NLRP inflammasome identified was NLRP1 (previously known as NALP1) (Martinon *et al.*, 2002), and it has been found expressed by both immune and non-immune cells (Kummer *et al.*, 2007). One of the main functions of NLRP1 is the protection against infection by *Bacillus anthracis* via detection of the anthrax lethal toxin (LT) (Levinsohn *et al.*, 2012). NLRP1 can also be activated by the bacterial wall muramyl dipeptide (MDP) (Hsu *et al.*, 2008). However, the mechanism involved in LT and MDP recognition by NLRP1 has not yet been elucidated, and no direct binding of these factors to NLRP1 has so far been demonstrated. It has therefore been proposed that an indirect mechanism of activation, possibly involving K^+ efflux or cathepsin B release into the cytoplasm may be involved in NLRP1 activation via these bacterial entities (Ali *et al.*, 2011).

The activation of NLRP6 inflammasome has surprisingly revealed a protective role in preserving host-microbiota homeostasis in the gastro-intestinal tract (Kempster *et al.*, 2011) as *in vivo* studies using models of intraperitoneal microbial infection in mice demonstrated that the NLRP6 inflammasome acts as negative regulator of innate immunity in pathogens clearance (Anand *et al.*, 2012). In contrast, NLRP7 has a 'classic' pro-inflammatory role: this inflammasome is involved in microbial lipopeptide recognition by macrophages, with subsequent systemic IL-1β release and induction of septic shock in a mouse model (Khare *et al.*, 2012). The NLRP12 inflammasome is among the least characterised inflammasomes, although a recent study by Vladimer *et al.* (2012) demonstrated a specific role for this inflammasome in the recognition of *Yersinia pestis* via promoting the release of both IL-1β and IL-18, as well as supporting lymphocyte activation and IFN-γ secretion. Another innate immune function of NLRP12 is that as a positive regulator of DC stimulation and myeloid cells migration during inflammation, as demonstrated *in vivo* in models of contact hypersensitivity (Arthur *et al.*, 2010).

In addition to the NLRP inflammasomes, the NLR inflammasome NLRC4 plays a key protective role against *Salmonella*, *Shigella*, *Pseudomonas*, and *Legionella* infections (Schroder and Tschopp, 2010). The most characterised NLRC4 inflammasome activator is the bacterial flagellin, but it has been suggested that also endogenous cell components can bind and activate NLRC4 (Mayor *et al.*, 2007). Moreover, there is evidence that K^+ efflux triggers NLRC4 inflammasome activation (Arlehamn *et al.*, 2010). However, is has been proposed that NLRC4 acts mostly as scaffolding protein and not as direct functional receptor: NLRC4 associates with various NAIP (NLR family-apoptosis inhibitory) proteins, and these NAIPs, rather than NLRC4, are involved in PAMPs ligand bindings, therefore they can oligomerise with NLRC4 to constitute a NAIP/NLRC4 inflammasome (Vance, 2015).

A second key inflammasome family is represented by the PYHIN members, annoverating the two inflammasomes absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16) (Dowling and O'Neill, 2012). AIM2 consists of a pyrin domain that recruits ASC and a DNA-binding HIN domain, whereas IFI16 has one pyrin domain and two HIN domains for DNA binding (Di Virgilio, 2013). The main function of these inflammasomes is the recognition of bacterial and viral dsDNA (Hornung *et al.*, 2009). The AIM2 protein possesses an N-terminal pyrin domain and a C-terminal HIN200 domain that recognises cytosolic dsDNA. DNA binding drives AIM2 heteroduplex formation, displacing the PYD domain (Jin *et al.*, 2012), and induces subsequent recruitment of ASC and caspase-1 into the complex (Hornung *et al.*, 2009). Activation of the AIM2 inflammasome also involves K^+ efflux, as demonstrated in macrophage activation during *Mycobacterium bovis* infection (Yang *et al.*, 2013). The IFI16 inflammasome has been named based on its ability to be induced by interferons. Similar to AIM2, it contains an N-terminal PYD domain, but it differs from AIM2 by two HIN domains with the consensus motifs A and B (Ludlow *et al.*, 2005). IFI16 has mostly a role in innate responses to nucleic acids (Unterholzner *et al.*, 2010), both double-stranded and single-stranded microbial DNA (Dowling and O'Neill, 2012).

The best characterised inflammasome, which recognises many different exogenous and endogenous triggers (Figure 1.6), is the NLRP3 inflammasome. As this thesis is

specifically focussed on investigating a role for complement-regulated NLRP3 inflammasome activity in T cell responses, the following sections in this chapter contain an in-depth description of NLRP3 activation, function and involvement in human diseases, with a final summarising look at the published evidences of complement-mediated NLRP3 regulation.

1.3.3 NLRP3 inflammasome structure and activation

Due to the plethora of activating signals and also disease conditions in which it is involved, the majority of currently published inflammasome studies focus on the NLRP3 inflammasome (Dowling and O'Neill 2012). The *NLRP3* gene (alternative names *NALP3*, *CIAS1*, *PYPAF1* or *cryopyrin*), coding for the NOD-like receptor NLRP3, has been identified originally by Hal Hoffman and collaborators (2001): they observed that mutations in this gene is associated with the development of severe auto-inflammayory conditions, specifically the familial cold autoinflammatory syndrome (FCAS) and the Muckle–Wells syndrome (MWS).
Similar to other NLRs, NLRP3 is composed of an amino-terminal pyrin domain, a central NACHT nucleotide-binding and oligomerization domain and carboxy-terminal LRRs (Figure 1.6) (Dowling and O'Neill, 2012). This discovery was followed few years later by the further characterisation of the NLRP3 inflammasome complex components: the NLRP3 molecule, the ASC adaptor protein and pro-caspase-1 (Agostini *et al.*, 2004). ASC is composed of a pyrin domain, which can recognise the pyrin domain of NLRP3 and a CARD domain that can interact with the CARD domain of pro-caspase-1 (Dowling and O'Neill, 2012).

In homeostatic conditions and resting cells, thus in absence of danger, the NLRP3 protein (as shown for other NLRs) is normally present in the cytoplasm in an inactive state in association with the heath shock protein 90 (HSP90) and the ubiquitin-ligase suppressor of the G2 allele of skp1 (SGT1) (Mayor *et al.*, 2007). NLRP3 inflammasome activation is induced upon sensing of specific danger signal molecules (such as bacterial peptidoglycan and monosodium urate particles): HSP90

and SGT1 release the NLRP3 protein, which then binds the Pyrin domain of ASC, leading to pro-caspase-1 binding to the CARD domain of ASC, via CARD–CARD homotypic interactions (Figure 1.7) (Mayor *et al.*, 2007; Dowling O'Neill, 2012). Once bound to this complex, ASC is converted to a prion-like shape with generation of long molecular filaments, crucial for inflammasome activation (Guo *et al*., 2015). This binding cascade triggers the formation of oligomers (known as "speck" complexes) of many molecules of NLRP3, ASC and pro-caspase-1, resulting in pro-caspase-1 autocatalysis and release the functional caspase-1 fragments p20 and p10; therefore, active caspase-1 is able to cleave the pro-inflammatory cytokines pro-IL-1β and pro-IL18 into their respective mature forms (Figure 1.7) (Dowling and O'Neill 2012).

Importantly, as part of a non-canonical inflammasome 'platform', NLRP3 can also bind to and activate other caspases aside from caspase-1, such as the human caspases-4 and caspase-5 (Schmid-Burgk *et al*., 2015; Baker *et al.*, 2015) and their mouse homolog caspase-11 (Rühl and Broz, 2015), in addition to caspase-8 (Gurung *et al.*, 2014). Moreover, the NLRP3 inflammasome can also induce the secretion of IL-1α, which lacks the recognition sequence for the protease activity of caspases, via a yet unidentified mechanism that does not require the proteolytic cleavage of IL-1α cytokine from a precursor form (Yazdi *et al.*, 2013).

The canonical NLRP3 inflammasome-driven caspase-1 activation and subsequent maturation of IL-1β and IL-18 has been mostly described both in human and in mice in myeloid innate immune cells, such as monocytes and macrophages which constitute the main source of IL-1β (Dinarello, 1996, Rao *et al.*, 2007). Furthermore a functional NLRP3 inflammasome has been observed recently in several non-immune cell types, such as microglia (Heneka *et al*., 2013), endothelial cells (Shahzad *et al*., 2015) and retinal pigment epithelial cells (Cao *et al.*, 2016). NLRP3 protein expression has been detected also in adaptive immune cells, such as in B and T lymphocytes (Kummer *et al.*, 2007) and more recently, a functional role for NLRP3 has been investigated in $CD4^+$ T lymphocytes in different studies: in a first study by Doitsh and collaborators (2014), where NLRP3 has been found expressed by $CD4^+$ cells were inflammasome activity was connected with pathogen clearance via a process involving a form of cell death called pyroptosis; however, this caspase-

1 dependent process required an inflammasome containing ASC but not NLRP3 *per se* and the exact mechanistic function of NLRP3 was not defined in this study. Subsequently, Bruchard *et al*. (2015) found that NLRP3 in the nucleus of mouse $CD4^+$ T cells can directly bind the DNA via the IRF4 transcription factor, therefore modulating expression of the genes associated with Th2 differentiation, including *Gata3*. Noteworthy, these two studies also did not describe a canonical functional NLRP3 inflammasome in T lymphocytes. Finally, a very recent work (published after this PhD thesis work was finalised) showed the presence of a functional non-canonical NLRP3/ASC/caspase-8 inflammasome in T cells, which regulates Th17 response in the murine model of MS, called experimental autoimmune encephalomyelitis (EAE) (Martin *et al.*, 2016). Noteworthy, before this experimental thesis work, a role for functional inflammasome in Th1 response had not been defined.

The NLRP3 inflammasome is activated by two main signals: 1) an upstream priming event (Signal 1), consisting of the engagement either of other PRRs, such as the Toll-like receptor 4 (TLR4) (whose principal ligands are lipopolysaccharides (LPS) derived from bacterial walls), or of cytokine receptors, such as the tumor necrosis factor receptor (TNFR), thus leading to NF-κB activation, nuclear translocation of this transcription factor and activation of the transcription of the *NLRP3* and *IL1B* genes, followed by their translation into proteins (Bauernfeind *et al*., 2009); 2) a secondary signal (Signal 2) required for functional inflammasome assembly and speck formation, driven by several exogenous or endogenous activators (Dowling and O'Neill, 2012). Exogenous stimuli driving Signal 2 and NLRP3 inflammasome assembly include many different microbial, fungal, viral and parasitic products (Dowling and O'Neill, 2012) such as the glycan MDP (Martinon *et al.*, 2004), toxins (Mariathasan *et al.*, 2006) and viral nucleic acid (Franchi *et al.*, 2014) and environmental polluters such as silica and asbestos (Yazdi *et al.*, 2010), in addition to other nanoparticles that can cause sterile inflammation (Di Virgilio, 2013), polyester (Demento *et al.*, 2009), titanium oxide (Yazdi *et al.*, 2010; Winter *et al.*, 2011), carbon (Reisetter *et al*., 2011), polystyrene (Lunov *et al.*, 2011), gold (Nguyen *et al.*, 2012), and silver (Yang *et al.*, 2012).

Moreover, the NLRP3 inflammasome can be activated by endogenous molecules that accumulate into complexes and cause severe diseases, for example cholesterol crystals in atherosclerosis (Samstad *et al.*, 2014), amyloid-β in Alzheimer disease (AD) (Heneka *et al.*, 2013), and monosodium urate (MSU) in gout (Cumpelik *et al.*, 2015). Other endogenous sources of Signal 2 are provided by events occurring during cell activation, including increased production of reactive oxygen species (ROS) (Zhou *et al.*, 2011), lysosomal damage and release of cathepsin B (Hornung *et al.*, 2008), increased cytoplasmic Ca^{2+} and K^{+} efflux (Yaron *et al*., 2015), changes in adenosine triphosphate (ATP) production or influx via P2X purinoreceptor 7 (P2X7) (Asgari *et al.*, 2013), and also products of glucose and lipid metabolism (reviewed in Arbore and Kemper, 2016).

Because many of these endogenous signals are derived from metabolic changes accompanying cell (hyper)activation, the NLRP3 inflammasome has now been recognised as a cellular sensor for metabolic activity and stress. This notion is much in line with the increasing understanding that the ancient PRRs (including complement and the TLRs) have all initially been identified as pathogen-fighting systems but clearly serve also in sensing self-derived danger and particularly metabolic and nutritional imbalances within cells and tissues (Schroder *et al.*, 2010). In line with a pro-inflammatory role, NLRP3 inflammasome priming and activation is driven by heightened glucose concentration, glucose metabolism and ATP production in several cells. Indeed, NLRP3 activity has been associated with type 2 diabetes, where the higher concentration of circulating glucose activates NLRP3 inflammasome; in this case activated NLRP3 cooperates with the thioredoxin-interacting protein (TXNIP), a protein linked to insulin resistance (Zhou *et al*., 2010). Further, enzymes and products of both glycolysis and the Krebs cycle partake in NLRP3 inflammasome regulation. Beginning with the first glycolytic enzyme hexokinase, Moon and colleagues (2015) have recently demonstrated that mTORC1 driven hexokinase 1 (HK1)-dependent glycolysis is a potent NLRP3 inflammasome inducer in macrophages. In addition, another key glycolytic enzyme, the pyruvate kinase M2, is involved in LPS-induced NLRP3 activation in macrophages by regulating the hypoxia-inducible factor 1 (HIF-1α), a transcription factor that binds to the promoter of *IL1B* gene and sustains the production of this cytokine (Palsson-

McDermott *et al.*, 2015). Furthermore, the Krebs cycle product succinate stabilises HIF-1α thus enhancing inflammasome function in macrophages (Tannahill *et al.*, 2013). As product of glycolysis and of increased cellular stress, ATP release is a strong driver of inflammasome assembly by activating the P2X7 receptor, therefore stimulating IL-1β and IL-18 cytokines release (Asgari *et al.*, 2013; Carta *et al.*, 2015). Also adenosine on its own is able to sustain NLRP3 inflammasome activation induced by LPS, where stimulation of the adenosin A(2A) receptor triggers the cAMP/PKA/CREB/HIF-1α pathway, then acting as a priming signal for *IL1B* transcription (Ouyang *et al.*, 2013).

Importantly, active NLRP3 has been found to colocalise with mitochondria in human monocytic cells, and mitochondrial activity (with increased metabolism and mitochondrial damage) produce ROS that sustain NLRP3 inflammasome function (Schroder *et al.*, 2010). In resting cells, homeostatic metabolism produces low intracellular ROS levels, however the oxidative stress associated with cellular infection or augmented metabolic demand to execute cell effector function causes intracellular accumulation of ROS. All known NLRP3 activators induce ROS and *in vitro* treatment with ROS scavengers blocks NLRP3 activation, therefore ROS are a more general indicator of cellular stress (Schroder *et al.*, 2010).

In addition to ROS production, mitochondrial damage leads also to diminished production of the co-enzyme NAD^+, and this further amplifies inflammasome activation. Reduced NAD^+ co-enzyme also affects the activity of the NAD^+-dependent deacetylase sirtuin 2 (SIRT2) involved in de-acetylation of α-tubulin: acetylated α-tubulin enhances microtubule polymerization in the cytosol and NLRP3 trafficking to mitochondria and inflammasome activation, as showed in macrophages in response to various inflammasome triggering stimuli (Misawa *et al*., 2013).

Conversely, several cell metabolites – and mostly those connected with resting, anergic or contracting immune cells – also inhibit NLRP3 inflammasome activity. For example, increased lactate, the main product of anaerobic glycolysis, is associated with reduced TLR4-mediated inflammasome induction in monocytes and macrophages, through a pathway dependent on the lactate receptor G protein-coupled receptor 81 (GPR81) and arrestin-β 2 (ARRB2) (Hoque *et al.*, 2014). Lipids and amino acids also inhibit inflammasome activity: AMP-dependent protein kinase (AMPK) is a key regulator of glucose homeostasis and energy balance, which

promotes the switch from energy-consuming processes to oxidative metabolism, associated with anti-inflammatory and homeostatic states, and favours mitochondrial biogenesis (O'Neill and Hardie, 2013). In line with a role for AMPK in negatively regulating the NLRP3 inflammasome, it has also been observed that AMPK activation driven by mono-unsatured fatty acids attenuate inflammasome function and IL-1β secretion *in vivo* in models of obesity (Finucane *et al.*, 2015). Other negative inflammasome regulators include: 1) the ketogenic metabolite β-hydroxybutyrate (an alternative source of ATP during energy-deficit status) prevents K^+ efflux, ASC oligomerization and specifically NLRP3 inflammasome speck formation, but not NLRC4 or AIM2 inflammasome activity (Youm *et al.*, 2015); 2) the activation of the N-methyl-d-aspartate (NMDA) receptor (that recognised polyunsaturated fatty acids and the amino acids aspartate and glutamate) which downregulates inflammasome activity in monocytes, macrophages and Kupffer cells via a β-arrestin dependent pathway (Farooq *et al.*, 2014); 3) the prostaglandin E2 (PGE2), a potent lipid mediator (Sokolowska *et al.*, 2015), which activates the PGE2 receptor subtype 4 (EP4) which increases intracellular cAMP and mediates blockage of NLRP3 activation (Lee *et al.*, 2013). Indeed cAMP can negatively regulate NLRP3 inflammasome via promoting NLRP3 ubiquitination by the E3 ubiquitin ligase MARCH7 (Membrane-Associated Ring Finger (C3HC4) 7) and subsequent NLRP3 degradation via the proteasome (Yan *et al.*, 2015).

Apart from cell metabolites, specific additional regulatory proteins modulate NLRP3 inflammasome function. The formation of the NLRP3 inflammasome in response to pathogenic microbes and other non-crystalline activators can be promoted by guanylate-binding protein 5 (GBP5) (Shenoy *et al.*, 2012). In addition, regulatory proteins can also disrupt the inflammasome speck formation. Belonging to the CARD-containing proteins that control caspase activity during cell death, CARD8 is a binding partner of NLRP3 (Agostini *et al.*, 2004), and proteins with a card domain can sequester pro-caspase-1 from its recruitment to a functional inflammasome complex. Moreover, the pyrin domain- only proteins POP1 and POP2 interfere with the pyrin–pyrin interaction among inflammasome components, such as NLRP3 and ASCs (Stehlik *et al.*, 2003; Dorfleutner *et al.*, 2007; Bedoya *et al.*, 2007). Noteworthy, this more complex regulatory mechanism might be present only in

humans, as genes encoding for COPs (CARD only proteins) and POPs have not yet been identified in the mouse (Di Virgilio, 2013). The inflammasome specks formation also requires the switch of ASC shape onto long filaments, and that has been shown to be regulated also by phosphorylation: upon NLRP3 inflammasome activation, ASC is phosphorylated specifically the kinases Syk and JNK (which have many different activating stimuli and many downstream targets apart from ASC), and inhibition of these kinases blocked speck formation and caspase-1 activation (Hara *et al*., 2013).

Finally, recent evidences suggest the possibility that inflammasome activation is transmitted from cell to cell: dying cells were reported to release ASC specks, resulting in the cleavage of extracellular pro-IL-1β and in activation of pro-caspase-1 in the macrophages internalising the specks. This mechanism of transmission of inflammation has serious implications for inflammatory diseases, as inflammasome ASC specks injected in vivo into mice are able to propagate the inflammation (Franklin *et al.*, 2014).

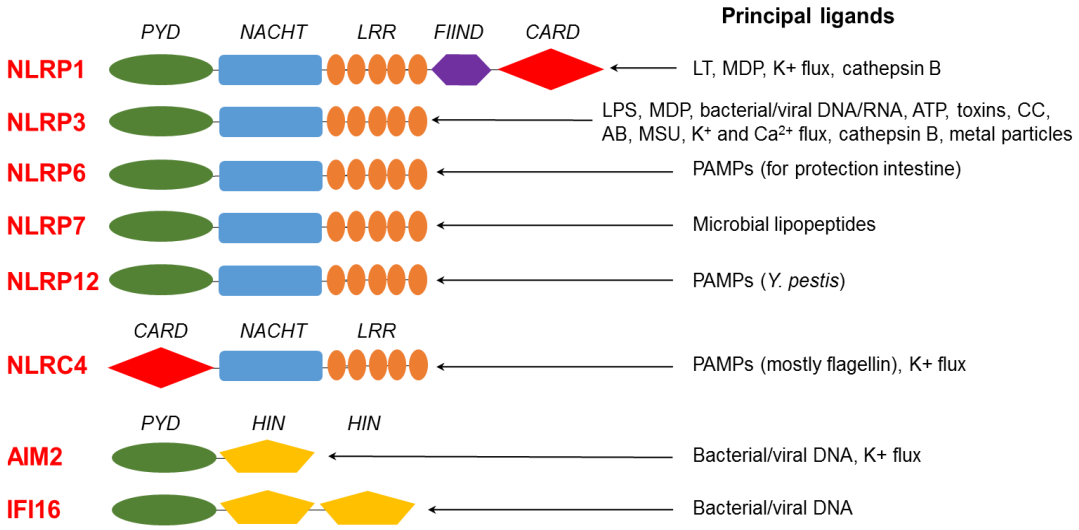


Figure 1.6 Schematic representation of the most characterised inflammasomes
Illustration of the structure of the main inflammasomes of the NLR family (NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4) and PYHIN family (AIM2, IFI16), with respective activating ligands (see text for details).
Abbreviations: PYD (pyrin domain), NACHT ((NAIP (neuronal apoptosis inhibitor protein), C2TA (class 2 transcription activator, of the MHC), HET-E (heterokaryon incompatibility) and TP1 (telomerase-associated protein 1)), LRR (leucine rich repeats), CARD (caspase recruitment domain), FIIND (function to find domain), HIN (hematopoietic interferon-inducible nuclear protein domain), LT (antrax lethal toxin), MDP (muramyl dipeptide), CC (cholesterol crystals), AB (amyloid-β), MSU (monosodium urate)

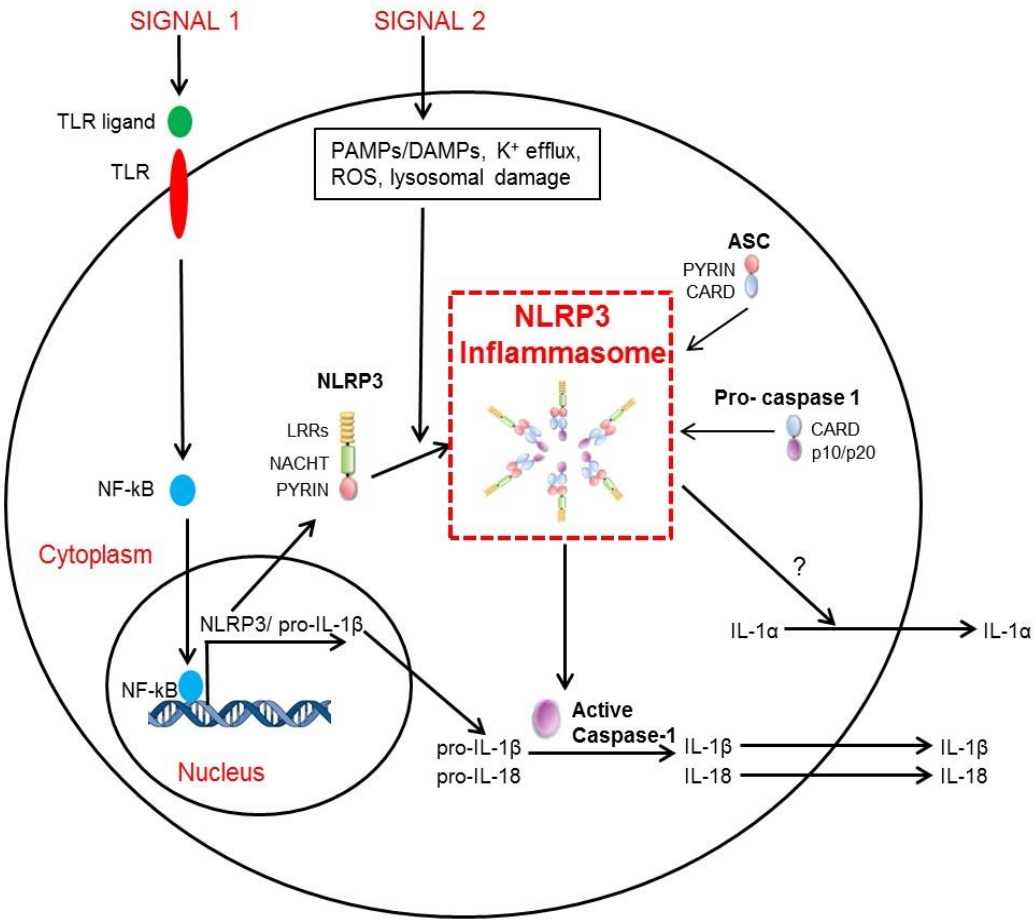


Figure 1.7 NLRP3 inflammasome structure and activation
Inflammasome activation requires two signals, a priming Signal 1 and an 'assembly' Signal 2. Signal 1: TLR ligands binds TLRs, leading to NF-kB nuclear translocation and activation of both NLRP3 and pro-IL1β transcription. Signal 2: exogenous microbial products (PAMPs) and endogenous DAMPs, and intracellular mechanisms such as ROS, K^+ efflux and lysosomal damage, activate NLRP3 (LLRs + NACHT + PYRIN domains), that interact with pro-caspase-1 (CARD + p10/p20) via the adaptor ASC (PYRIN + CARD), olygomerises and catalises the release of active caspase-1. Active caspase-1 cleaves the proteins pro-IL-1β and pro-IL-18 (the latter cytokine constitutively expressed), and mediates release of the active cytokines IL-1β and IL-18. Inflammasome regulates also the secretion of IL-1α from the cell, via a yet-unidentified caspase independent mechanism. More details are present above in the text.
Figure adapted from Dowling and O'Neill, 2012, used in Arbore MPhil upgrade thesis

1.3.4 NLRP3 inflammasome and human diseases

Apart from the important function as pathogen sensor, NLRP3 is a major sensor for cell metabolic activity and stress, and therefore not surprisingly, dysregulation of the NLRP3 inflammasome contributes to several metabolic diseases, such as type 2 diabetes (T2D), obesity, atherosclerosis, gout, colitis and cancer (reviewed in Menu and Vince, 2011), all conditions in which chronic inflammation plays a pivotal role.
T2D is a chronic inflammatory disease characterised by insulin resistance, elevated circulating levels of TNF, interleukins and adipokines, cytokine-like proteins released from the adipose tissue (Donath and Schoelson, 2011). IL-1β is involved in the pathogenesis of T2D via induction of insulin resistance and cellular functional impairments including dysregulation of mechanism that control apoptosis (Wen *et al.*, 2011). Neutralization of this cytokine by either the IL-1 receptor antagonist (IL-1RA) or anti-IL-1β antibodies improved control of glucose levels and cell function in clinical trials (Larsen *et al.*, 2007; Mandrup-poulsen *et al.*, 2010). Moreover, Lee and collaborators observed heightened NLRP3 inflammasome activity in myeloid cells from T2D patients when compared to those from unaffected individuals (Lee *et al.*, 2013), while mice lacking *Nlrp3*, *Asc* or *caspase-1* showed improved insulin sensitivity and glucose tolerance upon high fat diet (Zhou *et al.*, 2010; Wen *et al.*, 2011; Stienstra *et al.*, 2011; Vandanmagsar *et al.*, 2011; Stienstra *et al.*, 2012). NLRP3 inflammasome in T2D is activated by the increased levels of circulating glucose, thus stimulating IL-1β expression by pancreatic β-cells (Zhou *et al.*, 2010), and by saturated fatty acids such as palmitate increased through a high-fat diet, as palmitate inhibits the inflammasome negative regulator AMPK (Wen *et al.*, 2011). Thus, a diet rich in unsaturated fatty acids might ameliorate T2D, as these molecules are negative regulators of the NLRP3 inflammasome *in vitro* (L'homme *et al.*, 2013) and *in vivo*, including the polyunsaturated omega-3 fatty acids (Yan *et al.*, 2013).

Atherosclerosis is a pathology characterised by the progressive narrowing of the arterial vessels, due to altered lipid metabolism and accumulation of cholesterol crystals and white blood cells on the arterial wall, resulting in reduced flow of oxygen-rich blood to the organs (Weber and Noels, 2011). Low-density lipoproteins and free fatty acids, whose level is increased in the peripheral blood of

atherosclerosis patients, can induce TLRs therefore providing a priming signal for inflammasome activation (Masters *et al.*, 2011). Moreover the cholesterol crystals, which accumulate in atherosclerotic plaques, can activate the NLRP3 inflammasome through phagolysosomal damage and release of both cathepsin B and cathepsin L (Duewell *et al.*, 2010), and recent studies also associate cholesterol crystals with direct NLRP3 activation (Samstad *et al.*, 2014, Niyonzima *et al.*, 2015).

Gout is an inflammatory disease where MSU crystals accumulate in various tissues, specifically in the joints, inducing acute arthritis (So, 2008). Similarly, pseudogout is a pathology characterised by intra-articular deposition of calcium pyrophosphate dehydrate (CPPD) crystals (Martinon *et al*., 2006). Both MSU and CPPD strongly induce the NLRP3 inflammasome, in fact mice deficient for *Nlrp3*, *Asc*, and *caspase-1* have diminished release of IL-1β and IL-18 and are protected from inflammation in response to intraperitoneal injection of MSU or CPPD (Martinon *et al.*, 2006). Clinical trials on gout patients treated with drugs targeting IL-1β, such as Anakinra or Rilonacept, demonstrated high efficacy without substantial side effects (So *et al.*, 2007; Terkeltaub *et al.*, 2009), therefore therapies tackling also the NLRP3 inflammasome might be helpful to treat patients for which the use of colchicine, the standard gout therapy, is ineffective or contraindicated (Sundy, 2010).

Very recently, the NLRP3 inflammasome has also been associated with inflammatory bowel disease (IBD). IBD is a set of chronic inflammatory, relapsing-remitting conditions characterised by damage of the bowel wall, including ulcerative colitis (UC), a superficial colonic inflammation, and Crohn's disease (CD), a transmural inflammation that might affect any part of the gastro-intestinal tract (Neurath, 2014). SNPs in the *NLRP3* gene in humans have been associated with CD (Villani *et al.*, 2009), while increased IL‑1β production by lamina propria cells from CD patients has been detected (Youngman *et al.*, 1993). However, using mouse models of colitis induced with dextran sulphate (DSS) application, different groups found opposing results: NLRP3 knockout (KO) mice are either more susceptible to (Hirota *et al.*, 2011) or protected from (Bauer *et al.*, 2010) colitis. The underlying reason for these discrepancies may depend in large parts on differences in the gut microbiota in the different mouse strains used.

UC and CD predispose to colorectal cancer, where an excessive inflammatory response to commensal bacteria appears as the main cause of the neoplastic transformation of the intestinal epithelial cells (Menu and Vince, 2011). In line with a role for the NLRP3 inflammasome in the modulation of immune responses to bacteria and intestinal homeostasis, *in vivo* studies of colitis-associated cancer, induced by DSS and azoxymethane, demonstrated that NLRP3 inflammasome acts as negative regulator of tumorigenesis (Allen *et al.*, 2010). In any form of cancer, the inflammatory microenvironment is a key factor determining tumor progression. The contribution of dysregulated inflammasome activity to cancer is supported by the observation that several cancers are characterised by high release of IL-1β (Krelin *et al.*, 2007; Reed *et al.*, 2009; Dunn *et al.*, 2012) and concentration in biologic fluids of this cytokine is considered a negative prognostic indicator (Zitvogel *et al*., 2012). NLRP3 inflammasome hyperactivation has been observed in the interstitium of solid tumors, in concomitance with high concentration of extracellular ATP and expression of P2X7 receptor by tumor and infiltrating inflammatory cells (Okamoto *et al.*, 2010; Adinolfi *et al.*, 2012). Targeting IL-1β has been considered also a therapeutic strategy to fight cancer, however the IL-1β cytokine is also crucial in modulating anticancer-specific immunity. Moreover, NLRP3 has a detrimental role in tumor development that is independent from its canonical function as part of the inflammasome: Bruchard and collaborators (2015) observed that *Nlrp3* KO mice are protected from metastatic melanoma, where NLRP3 acts as transcription factor that facilitates the Th2 response, the latter favouring tumor progression. Thus, understanding the circumstances in which the NLRP3 inflammasome is protective or detrimental in the specific disease context will help to design specific therapeutic approaches to modulate NLRP3 activity in CD and colonic cancer.

Furthermore, NLRP3 inflammasome activity has been associated with diseases caused by particulate deposits, such as the neurological disorders MS, Alzheimer's diseases (AD), and age-related macular degeneration (AMD). MS is a pathology of the central nervous system (CNS) with currently no effective therapy. In MS, myelin-reactive $CD4^+$ T lymphocytes attack the oligodendrocytes and induce demyelination, with subsequent damage of neurotransmission, resulting in serious physical and mental impairments (Goverman, 2009). Several studies pointed out a

key role for the NLRP3 inflammasome in MS pathogenesis, because the NLRP3 inflammasome is known to participate in skewing human $CD4^+$ T cells toward the proinflammatory Th17 phenotype, driving inflammation in the CNS. Further, in clinical studies, higher levels of NLRP3, caspase-1 and IL-1β have been detected in peripheral blood mononuclear cells (PBMCs) from MS patients when compared with healthy controls (Peelen *et al.*, 2015). However, the role of the NLRP3 inflammasome in MS is still controversial, as different studies using the EAE *in vivo* model of MS, where disease is induced by immunization with CNS auto-antigens, showed opposing results: work by Gris and collaborators demonstrated a detrimental role for NLRP3 inflammasome in EAE, as *Nlrp3*$^{-/-}$ mice have a reduced disease severity and Th1 and Th17 responses to brain autoantigens (Gris *et al.*, 2010). Notwithstanding, it seems that the role of inflammasome in EAE is not intrinsic to T cells, as other researchers observed that $CD4^+$ T cells from EAE-induced WT and *Nlrp3*$^{-/-}$ mice delivered into the brain and spinal cord of *Rag2*$^{-/-}$ mice (which lack mature T cells) resulted in a similar degree of disease (Inoue *et al.*, 2012). Another study using *Nlrp3*$^{-/-}$ mice also failed to demonstrate a direct role for the NLRP3 inflammasome in EAE, while ASC participated in EAE progression by sustaining $CD4^+$ T cell survival (Shaw *et al.*, 2010). Most recently, a NLRP3-ASC inflammasome within $CD4^+$ lymphocytes has been found to regulate Th17 response in EAE model, via a pathway involving caspase-8 activation (Martin *et al.*, 2016), in opposition with the results from Inoue and colleagues (Inoue *et al*, 2012), where intrinsic NLRP3 inflammasome activity was not observed. Even though there are conflicting results, it seems that targeting the NLRP3 inflammasome with a specific small molecular inhibitor, the MCC950, strongly ameliorates EAE *in vivo* (Coll *et al.*, 2015, see below), although further studies are needed to translate these observations into clinical practice.

Alzheimer's disease (AD) is characterised by the accumulation of plaques of amyloid-β in the cerebrum (Heneka *et al.*, 2015). Amyloid-β was the first molecule found to be able to activate the NLRP3 inflammasome and IL-1β production *in vivo* in a mouse neurodegenerative disease model (Halle *et al.*, 2008). Heneka and collaborators also demonstrated that APP/PS1 mice (transgenic mice that develop chronic deposition of amyloid-β) with simultaneous NLRP3 deficiency have reduced

AD-like pathogenesis with diminished amyloid-β secretion, neuronal inflammation and cognitive damage (Heneka *et al.*, 2013).

Another disease that affects neurotransmission, specifically of vision, and that has been correlated with NLRP3 activity, is AMD (Gao *et al.*, 2015). The loss of vision in AMD is partially caused by the accumulation under the retina of molecular aggregates known as "drusen", composed by many components including protein, lipids, glycation products and complement, which are able to activate the inflammasome (Gao *et al.*, 2015). Some *in vivo* work has associated NLRP3 inflammasome activity with retinal epithelial cell degeneration and AMD progression (Tarallo *et al.*, 2012). However, another study has shown that NLRP3 inflammasome, activated by drusen components, might protect from the pathologic choroidal neovascularization typical of AMD (Doyle *et al.*, 2012). An in-depth understanding of the molecular mechanisms underlying the retinal cell death and the neoangiogenesis associated with AMD might allow to design therapies to modulate NLRP3 inflammasome function in AMD.

Importantly, the NLRP3 inflammasome has been implicated in the pathogenesis of several additional autoinflammatory syndromes, a group of genetically transmitted diseases characterised by recurrent fever attacks and systemic inflammation (Chitkara *et al.*, 2007). In contrast to autoimmune diseases, autoinflammatory disorders lack high-titre autoantibodies or increase in antigen-specific T cells (McDermott and Tschopp, 2007). The association of these diseases, including for example Behcet's disease (Bilginer *et al.*, 2010), TNF receptor-associated periodic syndrome and systemic juvenile idiopathic arthritis (Kalliolias and Liossis, 2008), with inflammasome activity is based by their favourable response to anti-IL-1β therapy (Menu and Vince, 2011; Masters *et al*., 2009).

In addition to these diseases, a specific set of hereditary autoinflammatory disorders, known as cryopyrin associated periodic syndrome (CAPS), are caused by over-active NLRP3 inflammasome due to a gain-of function mutation in the NLRP3 gene (Lachmann *et al.*, 2009). Because this thesis contains work on T cell isolated specifically from these patients, a more detailed description of this specific direct 'NLRP3-connected' syndrome is herein included.

1.3.5 Cryopyrin associated periodic syndrome

The discovery of the *NLRP3* gene by Hal Hoffman (Hoffman *et al.*, 2001) came as result of a mapping analysis of the genetic loci associated with a set of inherited aseptic autoinflammatory disorders known as "cryopyrinopathies" or "cryopyrin associated periodic syndromes" (CAPS). CAPS include the familial cold-induced autoinflammatory syndrome (FCAS, early on know as familial cold-induced urticaria), the Muckle-Wells syndrome (MWS, or also known as urticaria-deafness-amyloidosis syndrome, UDA), and neonatal onset multisystem inflammatory disorder (NOMID, also known as chronic infantile neurologic cutaneous and articular syndrome, CINCA) (Rigante *et al.*, 2016). These three rare diseases of childhood onset share clinical manifestations and are likely to represent a continuum of increasing severity during time/aging, where FCAS is the mildest and NOMID the most severe pathology (Kastner, 2005; Savic *et al.*, 2012). CAPS conditions can be traced to at least 170 different autosomal dominant mutations in the NLRP3 gene (Rigante *et al.*, 2016) and, in some cases, to somatic mutations appearing during fetal development (somatic mosaicism) (Tanaka *et al.*, 2011). All these mutations modify the normal structure of the NLRP3 protein, therefore increasing the tendency of NLRP3 towards spontaneous protein oligomerization and enhanced inflammasome activation resulting in uncontrolled and heightened IL-1β release (Savic *et al.*, 2012). The pathologically increased systemic production of this cytokine clinically manifests in recurrent outbreaks of periodic fever, skin rashes, fatigue, arthralgia, cold sensitivity, conjunctivitis and aseptic meningitis (Rigante *et al.*, 2016). Current therapies involve blockage of IL-1β via Anakinra, a soluble form of IL-1 receptor antagonist (IL-1RA), or Canakinumab, a monoclonal antibody against IL-1β (Menu and Vince, 2011; Lachmann *et al.*, 2009). The human disease can be replicated in two knock-in mouse models harboring two mutations associated with MWS and FCAS (Brydges *et al.*, 2009; Meng *et al.*, 2009). In particular the mouse model of MWS was generated by expression of the mutation *Nlrp3A350VneoR* (NLRP3A352V in the human NLRP3) in the myeloid lineage (crossed with LysMCre – meaning mice with the cre cDNA inserted onto the M lysozyme locus for the conditional expression in myeloid cells) - these MWS mice die in the

neonatal period and have heightened amount of circulating IL-1β and IL-18 (Brydges *et al.*, 2009). Using this MWS *in vivo* model, it has been recently demonstrated that targeting the NLRP3 inflammasome with the specific inhibitor MCC950 strongly ameliorates disease in mice (Coll *et al.*, 2015, see below). Moreover the same group demonstrated that MCC950 treatment resulted in lower *in vitro* inflammasome activation in PBMCs from CAPS patients. This latter study could be the basis for an alternative specific treatment of CAPS diseases via targeting directly the NLRP3 mutated protein and the overactive inflammasome activation rather than the cytokines produced as result of heightened inflammasome activity.

1.3.6 Inflammasome inhibitors and NLRP3 therapeutic targeting

The NLRP3 inflammasome is an attractive therapeutic target for ar range of diseases, including CAPS and the other examples mentioned above, where inappropriate NLRP3 activation contributes to the pathogenesis. However, effective activation of the NLRP3 inflammasome is fundamental in the host response to pathogens and likely also in enhancing the action of vaccine adjuvants, as the resulting products, IL-1β and IL-18 cytokines, modulate both innate and adaptive immune responses (Di Virgilio, 2013). A better understanding of the mechanism regulating the balance between beneficial and harmful NLRP3 inflammasome activity will help to design therapeutic approaches that target the deleterious effect of the inflammasome and simultaneously preserve its physiological protective functions in the host. To date, the mainstream therapies for CAPS and other chronic inflammatory pathologies aim at targeting IL-1β, including the drugs Anakinra, Canakinumab and Rilonacept (soluble decoy IL-1 receptor), while currently no therapies are in use that target IL-18 (Dinarello and van der Meer, 2013). The necessity of developing drugs that inhibit specifically the NLRP3 inflammasome rises from two main considerations: 1) targeting the inflammasome partly preserves the beneficial role of IL-1β in the protective responses to pathogen and endogenous danger; 2) another function of NLRP3 inflammasome, independent from IL-1β and IL-18 maturation, is the induction of pyroptosis, a type of inflammatory cell death that causes DAMPs

release therefore amplifying the inflammatory process, which plays also a key role in pathology of CAPS and other autoinflammatory diseases (Brydges *et al.*, 2013). Therefore, the possibility to specifically inhibit the NLRP3 inflammasome activation offers therapeutic promises.

Several small-molecule inhibitors targeting specifically NLRP3 have been described, such as parthenolide (Juliana *et al.*, 2010), CRID3 (Coll *et al.*, 2011), auranofin (Isakov *et al.*, 2014), isoliquiritigenin (Honda *et al.*, 2014), 3,4-methylenedioxy-β-nitrostyrene (He *et al.*, 2014), cyclopentenone prostaglandin 15d-PGJ2 (Maier *et al.*, 2015) and 25-hydroxycholesterol (Reboldi *et al.*, 2014), although their mechanism of action is not specific for NLRP3 and they do not exhibit substantial potency for *in vivo* usage. Even dimethylsulphoxide (DMSO) itself non-specifically inhibits inflammasome activation (Ahn *et al.*, 2014). A small-molecule inhibitor, glyburide, which is commonly used for treatment of T2D, was the first compound identified to be able to inhibit NLRP3 - but not NLRC4 and NLRP1 – inflammasome dependent IL-1β production; glyburide acts downstream of the P2X7 receptor and upstream of NLRP3 although via a yet unidentified mechanism (Lamkanfi *et al.*, 2009).

Most recently, a highly selective inhibitor of the NLRP3 inflammasome, the MCC950, has been reported (Coll *et al.*, 2015). MCC950 blocked both canonical (ATP, nigericin and MSU) and non-canonical (cytosolic LPS) NLRP3-dependent inflammasome activation at nanomolar concentrations, with no effect on NLRC4, NLRP1 or AIM2 inflammasomes. As mentioned above, *in vivo* MCC950 has been shown to reduce IL-1β production and to attenuate the severity of the EAE model of MS disease. MCC950 administration also rescued the neonatal lethality in a mouse model of CAPS (Coll *et al.*, 2015). The mechanism of action of MCC950 is not fully understood, although its inhibition of inflammasome activity has been observed in association with reduced NLRP3/ASC speck formation. MCC950 is a small diarylsulfonylurea molecule, much cheaper to synthesise when compared to the recombinant antibodies and proteins currently used as therapeutics, therefore clinical trials are warranted to define whether this compound can be used as a more effective and cost-efficient therapy for NLRP3 inflammasome-associated inflammatory diseases (Coll *et al.*, 2015).

1.3.7 Complement-mediated NLRP3 inflammasome regulation

The involvement of complement in inflammasome modulation is not surprising, as these systems share a 'mutual interest' in protecting the host against danger: the complement system is evolutionarily among the most ancient PRR system (Köhl, 2006) and there is an acknowledged strong cross-talk between complement and other PRRs such as TLRs (Zhang *et al.*, 2007). Recently, complement activators, receptors and/or regulatory molecules, either independently or in conjunction with incoming signals from TLRs or RIG-Is, have emerged as additional key modulators of NLRP3 inflammasome function (reviewed in Arbore and Kemper, 2016). Complement-derived inflammasome modulators include molecules at the beginning of the complement cascade, such as C1q, terminal complement activation products such as the MAC complex, the receptors for the anaphylatoxins C3a and C5a and possibly also the co-stimulatory molecule CD46 (the receptor for C3b) (Figure 1.8).

A functional connection between complement and inflammasome activity had been suggested before the inflammasome machinery was discovered: studies already in the 1980s by Haeffner-Cavallion and colleagues observed that the anaphylatoxin C3a induces IL-1β production by human monocytes (Haeffner-Cavallion *et al.*, 1987). More recently, it has been demonstrated that locally produced C3a, which binds in an autocrine fashion to the C3aR expressed by human monocytes, increases ATP efflux from the monocyte cytosol (through ERK1/ERK2 phosphorylation and modulation of a yet-unidentified channel) (Asgari *et al.*, 2013). Therefore, the NLRP3 inflammasome, primed by LPS binding TLR4 *in vitro* in monocytes, is activated via this C3a-driven ATP efflux through activation of the ATP receptor P2X7, a potent Signal 2 for NLRP3 inflammasome activation (Mariathasan *et al.*, 2006), and contributes to substantially increased mature IL-1β production by monocytes (Asgari *et al.*, 2013).

Also, signaling through the anaphylatoxin C5a drives both Signal 1 and Signal 2 for NLRP3 inflammasome activation (Arbore and Kemper, 2016). Regarding a role for C5a in NLRP3 priming, Samstad *et al*. (2014) showed that C5a generated by human monocytes exposed to cholesterol crystals (CC) (which activate both the classical - via C1q - and the alternative complement pathways), together with TNF-α, primes

the NLRP3 inflammasome by increasing *IL1B* transcription in PBMCs. Importantly, the C5a-regulated NLRP3 activity has impact on the development of human diseases, such as atherosclerosis, where high density lipoproteins might be beneficial as they reduce CC-induced complement activation and subsequently diminish NLRP3 inflammasome activity in human monocytes and granulocytes (Niyonzima *et al.*, 2015). C5a signaling seems to regulate *IL1B* gene expression via induction of NF-κB, as demonstrated in non-immune retinal pigment epithelial cells in the context of AMD, another disease in which both complement and inflammasome activation have been implicated (Cao *et al.*, 2016). C5a delivers not only a priming Signal 1 but also the Signal 2 for NLRP3 inflammasome activation. In models of gout disease, C5aR1 activation on monocytes potentiates NLRP3 inflammasome induction by uric acid crystals, associated with augmented intracellular Ca^{2+}, efflux of K^{+}, lysosomal damage and cathepsin B release (An *et al.*, 2014); this role for C5a has been also observed in neutrophils, using a mouse model of peritonitis with injection of MSU to induce gout, and in human exudates from the articular joints of gout patients (Cumpelik *et al.*, 2015). C5a may activate the NLRP3 inflammasome by increasing ROS generation, a general danger signal triggering the inflammasome (Schroder *et al.*, 2010): ROS production has been linked tightly with C5a anaphylatoxin receptor activation in granulocytes and monocytes (Guo *et al.*, 2003; Daniel *et al.*, 2006), and this C5a-mediated ROS release drives the NLRP3 inflammasome function in PBMCs (Samstad, et al., 2014). A role for C5aR1 in ROS generation and subsequent induction of the NLRP3 inflammasome is further supported by the fact that C5aR1 activation drives PI3K signaling (Strainic *et al.*, 2008), which also acts upstream of mitochondrial ROS production and NLRP3 inflammasome activation (Ives *et al.*, 2015).

In analogy with pore forming toxins which are strong inflammasome inducers (Mariathasan *et al.*, 2006), sublytic deposition of the membrane pore-forming MAC is also able to activate the NLRP3 inflammasome: in murine DCs, sublytic MAC elicits the caspase-1 dependent secretion of IL-1β and IL-18 (Laudisi *et al.*, 2013); similarly, in human lung epithelial cells, MAC formation results in increased intracellular Ca^{2+} fluxes from the endoplasmic reticulum and loss of mitochondrial

transmembrane potential due to Ca^{2+} accumulation in the mitochondrial matrix, and this process triggers NLRP3 inflammasome activation (Triantafilou *et al.*, 2013).

In line with the fact that the complement system is involved in both initiation and contraction of immune responses (Yamamoto *et al.*, 2013), another complement component, the classic pathway molecule C1q, appears to negatively regulate NLRP3 inflammasome activity. This effect has been demonstrated in human macrophages where C1q suppresses caspase-1 activation and mature IL-1β production during the phagocytosis of apoptotic lymphocytes (Benoit *et al.*, 2012). The authors suggest that C1q may indirectly inhibit the inflammasome via positively regulating NLRP12 mRNA expression and IL-10 secretion, and both NLRP12 (via NF-kB suppression) and IL-10 (through JAK signaling activation) negatively modulates NLRP3 inflammasome activation (Kim *et al.*, 2004a; Williams *et al.*, 2005). Thus, presumably C1q acts as a rheostat in controlling the excessive NLRP3 inflammasome activation caused by the DAMPs derived from late apoptotic cells (Benoit *et al.*, 2012).

In sum, a picture emerges were complement activation (at distinct locations) clearly intersects with the activation and also control of the NLRP3 inflammasome – albeit the exact molecular mechanisms underlying this functional crosstalk remain yet to be defined.

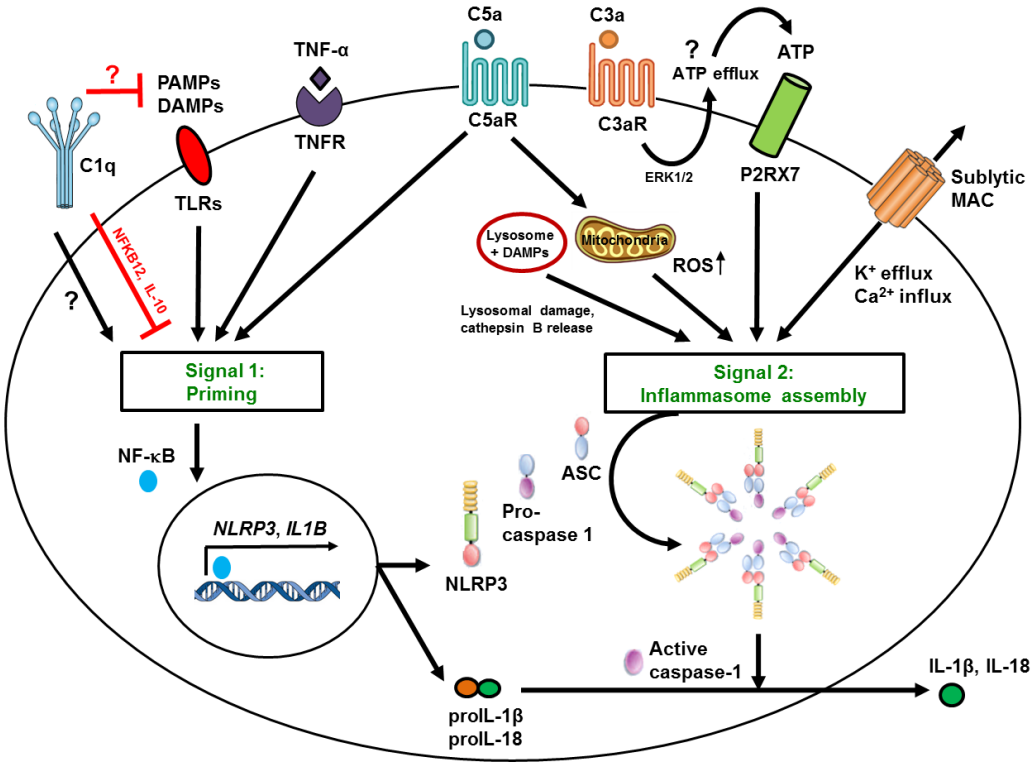


Figure 1.8 Complement-mediated NLRP3 inflammasome regulation
NLRP3 inflammasome activation requires two distinct signals. The 'priming' Signal 1 is triggered by PAMP/DAMP recognition by PPRs (for example TLRs) and certain cytokines (TNF-α) and drives NF-κB nuclear translocation and *NLRP3* and *IL1B* gene transcription. Signal 2 induces the assembly of NLRP3, ASC and caspase-1 supracomplex to form an active NLRP3 inflammasome, where active caspase-1 processes pro-IL-1β into mature IL-1β. The complement components C1q and C5aR1 (together with TNFR and/or TLR signaling) potentiate Signal 1. C5aR1 act as a priming signal to sustain inflammasome activation during the uptake of DAMPs, with a mechanism involving increased lysosomal damage and cathepsin B release. C5aR1 activation directly delivers Signal 2 for NLRP3 inflammasome activation, via induction of mitochondrial damage and intracellular accumulation of ROS. The C3aR regulates ATP efflux (via a not yet identified channel, denoted by a question mark) and subsequent autocrine P2X7 engagement, and sublytic MAC formation increases intracellular Ca^{2+} levels and mitochondrial membrane potential. Of note, C1q can increase canonical NLRP3 inflammasome activation in epithelial cells (Doyle *et al.*, 2012) through a not yet defined mechanism (denoted by a question mark) but can also function as a negative regulator of NLRP3 inflammasome activation by sequestering DAMPs (such as cholesterol crystals) and inhibiting PPRs signaling.
From Arbore and Kemper, 2016.

1.4 Hypothesis and aims of the thesis

The intracellular activation of C3 into C3a and C3b by CTSL and the subsequent engagement of C3 activation fragment receptors C3aR and CD46 in an autocrine fashion are critical events in normal human Th1 response induction and contraction (Cardone *et al.*, 2010; Liszewski *et al.*, 2013). Similary, the role of C5 activation products as key drivers in immunological responses is undisputed (Woodruff *et al.*, 2011). Although there is some literature published that indicates a direct role of C5aR signaling on mouse $CD4^+$ T cells, the data obtained so far are still controversial. Further, the existence of an 'intracellular C5 system' and its potential role in the regulation of human Th1 immunity has not yet been defined.
Based on the preliminary observation that exogenous addition of C5a and C5adesArg to *in vitro* activated human $CD4^+$ lymphocytes affects IFN-γ production severely and these cells indeed express both C5aR1 and C5aR2 (Claudia Kemper and Antonella Fara), the overarching aim of this thesis work was to address the initial hypothesis that:

'Human $CD4^+$ T cells harbor (aside from an intracellular C3 system) also an 'intracellular C5 system' and this system contributes to normal Th1 effector responses.'

The initial set of experiments herein reported demonstrated that this hypothesis was correct and that intracellular C5a generation and autocrine C5aR1 and C5R2 activity are indeed also required for normal human Th1 induction (IFN-γ secretion) and contraction. Further experimental work addressed the molecular mechanism underlying C5-driven support of IFN-γ production in human $CD4^+$ T cells. Based on data derived from gene arrays using human $CD4^+$ T cells that had been activated in the presence of C5aR-modulating agents, the following second aim/hypothesis was developed:

'Normal human Th1 immunity requires the intrinsic activity of a C5-regulated canonical NLRP3 inflammasome.'

Chapter 2

Results and Discussion Part 1:

T helper 1 immunity requires complement-driven, NLRP3 inflammasome activity in $CD4^+$ T cells

2.1 Summary of results

As outlined above, and based on preliminary data generated in the Kemper laboratory, the initial hypothesis of this thesis work was that:

1. ***'Human CD4$^+$ T cells harbor (aside from an intracellular C3 system) also an 'intracellular C5 system' and this system contributes to normal Th1 effector responses'***

and

2. ***'Normal human Th1 immunity requires the intrinsic activity of a C5-regulated canonical NLRP3 inflammasome'***

The experiments reported in this thesis, which I performed also in collaboration with researchers coming from different institutions (section 2.5), demonstrated the validity of these two hypotheses and the work has been published in 2016 in *Science* as a research article entitled "T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4$^+$ T cells" (Arbore *et al.*, 2016).

The published paper (sections 2.2, 2.3 and 2.4) is incorporated into the pages 83 to 116 of this thesis. The following points summarise the key findings of the paper:

1. C5 storages and constitutive C5a generation in resting and activated human CD4$^+$ T cells was demonstrated *in vitro* by means of flow cytometry staining and confocal microscopy. The expression levels and (sub)cellular localization of the C5aR1 and C5aR2 were confirmed via RT-PCR, immunoblotting, flow cytometry staining, confocal analysis and binding assays using radioactively labelled C5a, the latter in collaboration with Professor Andreas Klos (University of Hannover, Germany), and demonstrated that resting and activated CD4$^+$ T cells express the C5aR1 exclusively intracellularly while the C5aR2 is expressed both intracellularly and on the cell surface.

2. Using siRNA technique to reduce intracellular C5aR1 expression, a cell non-permeable C5aR1/C5aR2 double antagonist (which blocked only C5aR2 activity on the T cell surface as the C5aR1 is only expressed intracellularly), and a specific cell non-permeable C5aR2 agonist (provided by Professor Jörg Köhl, University of Lübeck, Germany; Dr. Trent Woodruff, University of Queensland, Australia; and Dr. Peter Monk, University of Sheffield, UK), it has been demonstrated that the engagement of intracellular C5aR1 by intracellular C5a (driven by TCR activation and CD46 co-stimulation) is required for induction of normal levels of IFN-γ (but not IL-4) and that the cell surface expressed C5aR2 (engaged by secreted C5a/C5adesArg in an autocrine fashion) negatively regulates IFN-γ production in human $CD4^+$ T cells.
3. Subsequent gene array analyses (in collaboration with Dr. Paul Lavender and Dr. Behdad Afzali at KCL) using α-CD3 + α-CD46 versus α-CD3 + α-CD46 + dRA activated human $CD4^+$ T cells revealed unexpected *NLRP3* and *IL1B* gene expression upon in vitro activation, which was further increased for *IL1B* by dRA treatment. Subsequent work using $CD4^+$ T cells from healthy donors and a range of methods (immunoblotting, mRNA PCR, confocal microscopy and FACS analyses) revealed the activation of a canonical NLRP3 inflammasome in T cells with subsequent activation of caspase-1 cleavage and generation of mature IL-1β (but not IL-18). Further, NLRP3-driven autocrine IL-1β is required for normal levels of IFN-γ secretion, hence Th1 induction.
4. In collaboration with Dr Helen Lachmann from the National Amyloidosis Centre (Royal Free Hospital, UCL, London) and Professor Andy Cope (King's College London), which provided blood specimens of CAPS patients, it has also been shown that intrinsic NLRP3 inflammasome activity and thus Th1 responses are hyperactive in $CD4^+$ lymphocytes isolated from these patients but that these can be normalised *in vitro* by specific inhibition of the NLRP3 inflammasome with MCC950 (provided by Professors Matthew Cooper and Luke O'Neill).
5. Mechanistically, CD46 co-stimulation during TCR activation increases *NLRP3* and *IL1B* gene expression (Signal 1), while intracellular C5aR1 activation drives mitochondrial ROS production (Signal 2) that leads to the assembly of the NLRP3 inflammasome and IL-1β secretion supporting Th1 induction. The

mechanism by which surface C5aR2 negatively regulates this process is not yet clear but could include a direct negative effect on intracellular C5aR1 signaling or a separate, yet undefined regulative pathway.

6. In collaboration with Dr. Erin West, Professor Warren Leonard, Dr. Katrin Mayer-Barber and Professor Alan Sher (all NIH, USA, MD), the *in vivo* significance of this novel 'C5-NLRP3-inflammasome' axis in $CD4^+$ T cells for normal Th1 responses was proved: T cells isolated from *Nlrp3*$^{-/-}$ mice had significantly reduced anti-viral $CD4^+$ T cell IFN-γ responses in a model of lymphocytic choriomeningitis virus (LCMV) infection and also led to more severe disease in T cell transfer models of colitis and graft versus host disease (GvHD), as the reduction in Th1 responses induced unrestrained Th17 activity in these animals.

In sum, this thesis work demonstrated that 'the regulated crosstalk between intracellularly activated complement components ('complosome') and the NLRP3 inflammasome is fundamental to normal Th1 induction and regulation (Figure 2.1).

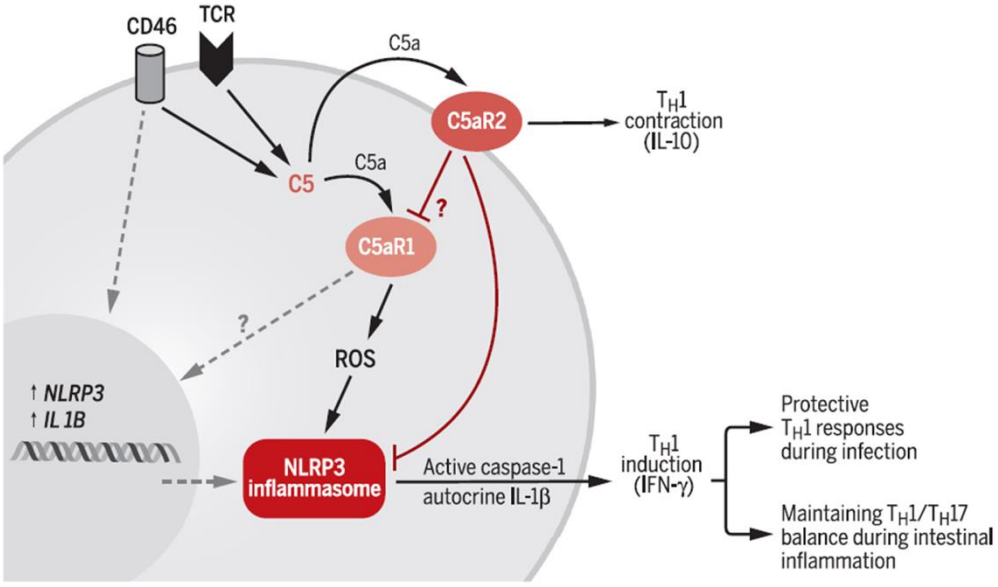


Figure 2.1 An intrinsic complement-NLRP3 axis regulates human Th1 responses
T cell receptor activation and CD46 costimulation trigger NLRP3 expression and intracellular C5a generation. Subsequent intracellular C5aR1 engagement induces ROS production (and possibly *IL1B* gene transcription) and NLRP3 assembly, which in turn mediates IL-1β maturation. Autocrine IL-1β promotes Th1 induction (IFN-γ production) but restricts Th1 contraction (IL-10 coexpression). C5aR2 cell surface activation by secreted C5a negatively controls these events via undefined mechanisms. Dysfunction of this system contributes to impaired Th1 responses in infection or increased Th17 responses during intestinal inflammation.
From Arbore *et al.*, 2016

2.2 Research article summary

(Arbore *et al*., Science, 2016)

RESEARCH ARTICLE SUMMARY

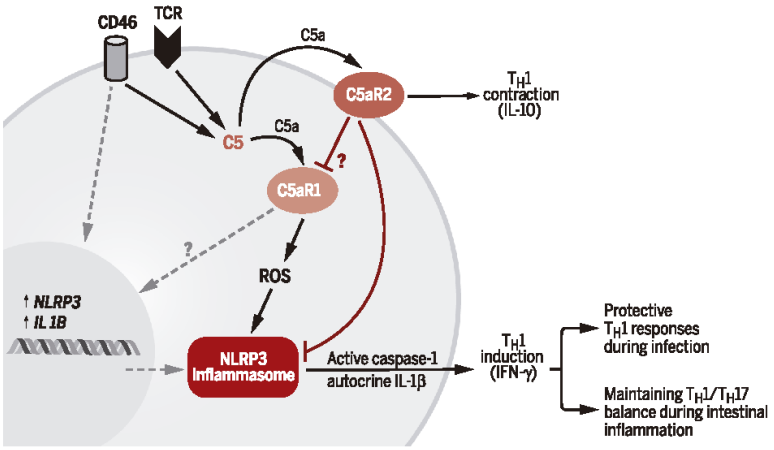
IMMUNE REGULATION

T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4$^+$ T cells

Giuseppina Arbore,* Erin E. West,* Rosanne Spolski, Avril A. B. Robertson, Andreas Klos, Claudia Rheinheimer, Pavel Dutow, Trent M. Woodruff, Zu Xi Yu, Luke A. O'Neill, Rebecca C. Coll, Alan Sher, Warren J. Leonard, Jörg Köhl, Pete Monk, Matthew A. Cooper, Matthew Arno, Behdad Afzali, Helen J. Lachmann, Andrew P. Cope, Katrin D. Mayer-Barber, Claudia Kemper†

INTRODUCTION: The inflammasomes and the complement system are traditionally viewed as quintessential components of innate immunity required for the detection and elimination of pathogens. Assembly of the NLRP3 inflammasome in innate immune cells controls the maturation of interleukin (IL)–1β, a proinflammatory cytokine critical to host defense, whereas activation of the liver-derived complement key components C3 and C5 in serum leads to opsonization and removal of microbes and induction of the inflammatory reaction. Recent studies, however, have highlighted an unanticipated direct role for complement C3 also in human T cell immunity: The anaphylatoxin C3a receptor (C3aR) and the complement regulator CD46 (which binds C3b) are critical checkpoints in human T cell lineage commitment, and they control initiation and resolution of T helper 1 (T_H1) responses in an autocrine fashion via T cell–derived and intracellularly activated C3. We explored a novel functional cross-talk of complement with the NLRP3 inflammasome within CD4$^+$ T cells and determined how the cooperation between these two "classically" innate systems directly affects interferon-γ (IFN-γ) production by adaptive immune cells.

RATIONALE: Given the critical role of intracellular C3 activation in human T_H1 responses and the importance of C5 activation products in inflammation, we investigated whether human CD4$^+$ T cells also harbor an "intracellular C5 activation system" and by what means this system may contribute to effector responses by using C5aR1 and C5aR2 agonists and antagonists, T cells from patients with cryopyrin-associated periodic syndromes (CAPS), and mouse models of infection and autoimmunity.



An intrinsic complement-NLRP3 axis regulates human T_H1 responses. T cell receptor activation and CD46 costimulation trigger *NLRP3* expression and intracellular C5a generation. Subsequent intracellular C5aR1 engagement induces ROS production (and possibly *IL1B* gene transcription) and NLRP3 assembly, which in turn mediates IL-1β maturation. Autocrine IL-1β promotes T_H1 induction (IFN-γ production) but restricts T_H1 contraction (IL-10 coexpression). C5aR2 cell surface activation by secreted C5a negatively controls these events via undefined mechanisms. Dysfunction of this system contributes to impaired T_H1 responses in infection or increased T_H17 responses during intestinal inflammation.

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RESULTS: Human CD4$^+$ T cells expressed C5 and generated increased intracellular C5a upon T cell receptor activation and CD46 autocrine costimulation. Subsequent engagement of the intracellular C5aR1 by C5a induced the generation of reactive oxygen species (ROS) and the unexpected assembly of a functional NLRP3 inflammasome in CD4$^+$ T cells, whereas the surface-expressed C5aR2 negatively controlled this process.

NLRP3 inflammasome–dependent autocrine IL-1β secretion and activity were required for optimal IFN-γ production by T cells; consequently, dysregulation of NLRP3 function in these cells affected their normal effector responses. For example, mutated, constitutively active NLRP3 in T cells from patients with CAPS induced hyperactive T_H1 responses that could be normalized with a NLRP3 inhibitor. The in vivo importance of a T cell–intrinsic NLRP3 inflammasome was further supported by the finding that IFN-γ production by *Nlrp3*$^{-/-}$ CD4$^+$ T cells was significantly reduced during viral infections in mice and that diminished T_H1 induction due to lack of NLRP3 function in a CD4$^+$ T cell transfer model of colitis led to uncontrolled T_H17 infiltration and/or expansion in the intestine and aggravated disease.

CONCLUSION: Our results demonstrate that the regulated cross-talk between intracellularly activated complement components (the "complosome") and the NLRP3 inflammasome is fundamental to human T_H1 induction and regulation. The finding that established innate immune pathways are also operative in adaptive immune cells and orchestrate immunological responses contributes to our understanding of immunobiology and immune system evolution. In addition, the results suggest that the complement-NLRP3 axis in T cells represents a novel therapeutic target for the modulation of T_H1 activity in autoimmunity and infection. ■

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2.3 Research article main text (Arbore *et al*., Science, 2016)

RESEARCH ARTICLE

IMMUNE REGULATION

T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4$^+$ T cells

Giuseppina Arbore,[1]* Erin E. West,[2]* Rosanne Spolski,[2] Avril A. B. Robertson,[3] Andreas Klos,[4] Claudia Rheinheimer,[4] Pavel Dutow,[4] Trent M. Woodruff,[5] Zu Xi Yu,[5] Luke A. O'Neill,[6] Rebecca C. Coll,[3] Alan Sher,[7] Warren J. Leonard,[2] Jörg Köhl,[8,9] Pete Monk,[10] Matthew A. Cooper,[3] Matthew Arno,[11] Behdad Afzali,[1,12] Helen J. Lachmann,[13] Andrew P. Cope,[14] Katrin D. Mayer-Barber,[15] Claudia Kemper[1,2]†

The NLRP3 inflammasome controls interleukin-1β maturation in antigen-presenting cells, but a direct role for NLRP3 in human adaptive immune cells has not been described. We found that the NLRP3 inflammasome assembles in human CD4$^+$ T cells and initiates caspase-1–dependent interleukin-1β secretion, thereby promoting interferon-γ production and T helper 1 (T_H1) differentiation in an autocrine fashion. NLRP3 assembly requires intracellular C5 activation and stimulation of C5a receptor 1 (C5aR1), which is negatively regulated by surface-expressed C5aR2. Aberrant NLRP3 activity in T cells affects inflammatory responses in human autoinflammatory disease and in mouse models of inflammation and infection. Our results demonstrate that NLRP3 inflammasome activity is not confined to "innate immune cells" but is an integral component of normal adaptive T_H1 responses.

The complement system is an ancient innate immune sensor system that is essential for elimination of pathogens by the host. Processing in serum of liver-derived C3 into C3a and C3b and of C5 into C5a and C5b activation fragments leads to opsonization and removal of invading microbes, mobilization of innate immune cells, and induction of inflammatory reactions (*1*). However, complement also profoundly regulates adaptive immunity: In addition to T cell receptor (TCR) activation, costimulation, and the presence of interleukin (IL)–12 (*2*), human CD4$^+$ T cells also depend on the activation of T cell–expressed complement receptors binding C3 activation fragments for normal T helper 1 (T_H1) induction (*3*). Unexpectedly, the engagement of complement receptors on T cells is independent of systemic complement but instead is mediated in an autocrine manner by complement activation fragments produced by the T cell itself. In particular, C3a and C3b are generated intracellularly via cathepsin L–mediated cleavage of C3 in T cells upon TCR activation (*4*). These engage their respective receptors—a G protein–coupled receptor (GPCR) C3a receptor (C3aR) and the complement regulator CD46 (which binds C3b)—and induce autocrine interferon-γ (IFN-γ) (*5*, *6*). Mechanistically, C3aR- and CD46-mediated signals (i) regulate IL-2R assembly, (ii) up-regulate the glucose transporter GLUT1 and the amino acid transporter LAT1, and (iii) up-regulate mTORC1 activation, which is required for the metabolic programming essential for IFN-γ induction (*7*).

However, CD46 costimulation is not only essential for IFN-γ production and human T_H1 induction; it also contributes to the negative control of T_H1 responses. Together with IL-2, CD46-mediated signals drive the coexpression of immunosuppressive IL-10 in T_H1 cells and initiate their switch into a (self-)regulatory and contracting phase (*3*). Accordingly, C3- and CD46-deficient patients suffer from recurrent infections and have severely reduced T_H1 responses in vitro and in vivo, whereas T_H2 responses remain intact (*5*, *8*). Conversely, uncontrolled intracellular C3 activation (or dysregulated CD46 engagement) in T cells contributes to hyperactive T_H1 responses observed in autoimmunity (*3*, *4*, *9*) that can be normalized pharmacologically by targeting intracellular cathepsin L function (*4*). Of note, CD46 is not expressed on somatic tissue in rodents and a functional homolog has not yet been identified. This indicates the existence of substantial differences in the complement receptor–driven pathways regulating T cell responses between species [reviewed in (*6*)].

Given the critical role of intracellular C3 processing in human T_H1 induction and contraction and the importance of C5a generation in inflammation, we investigated whether human CD4$^+$ T cells also harbor an "intracellular C5 activation" system contributing to effector responses.

Autocrine activation of C5a receptors regulates IFN-γ production by human CD4$^+$ T cells

Human CD4$^+$ T lymphocytes isolated from healthy donors contained intracellular stores of C5 and produced low levels of C5a in the resting state. TCR activation, in particular TCR + CD46 costimulation, increased the amounts of intracellular C5a, and this was associated with the secretion of C5a to the cell surface (Fig. 1, A and B). C5a, as well as the C5a "des-arginized" form of C5a (C5adesArg) generated by carboxypeptidase processing, can bind two distinct GPCR receptors, C5aR1 (CD88) and C5aR2 (GPR77, C5L2) (*10*, *11*). Binding of C5a to C5aR1 preferentially mediates proinflammatory responses. The function of C5aR2 varies with cell type; C5aR2 can act either as a nonsignaling decoy receptor antagonizing C5aR1 or as an active transducer of pro- or anti-inflammatory signals (*11*–*14*).

Both extra- and intracellular localization of C5aR1 and C5aR2 on human monocytes have been reported (*14*, *15*), but expression patterns in human CD4$^+$ T cells have not been described in detail. We detected expression of both *C5AR1* and *C5AR2* mRNA in human CD4$^+$ T cells (Fig. 1C) and protein by immunoblotting (fig. S1A), confocal microscopy (Fig. 1D), and flow cytometry (Fig. 1, E and F). Although mRNA amounts for *C5aR1* and *C5aR2* vary in T cells (Fig. 1C) (*16*), the protein levels for these receptors are comparable among donors (Fig. 1E). In resting and activated CD4$^+$ T cells, C5aR1 is expressed exclusively intracellularly and in low amounts, whereas the C5aR2 receptor is abundantly present inside and to a lesser degree on the cell surface (Fig. 1F). Using human embryonic kidney (HEK) 293 cells that had been stably transfected to express C5aR1, C5aR2, or no receptor, we corroborated the specificity of reagents used for C5a receptor detection (fig. S1, B and C). Competitive binding studies of C5a labeled with radioactive ^{125}I (Fig. 1G and fig. S1D) confirmed the ability of resting and activated human CD4$^+$ T cells to bind C5a.

To determine whether autocrine engagement of the C5a receptors on T cells regulates T_H1 induction, we activated human CD4$^+$ T cells with

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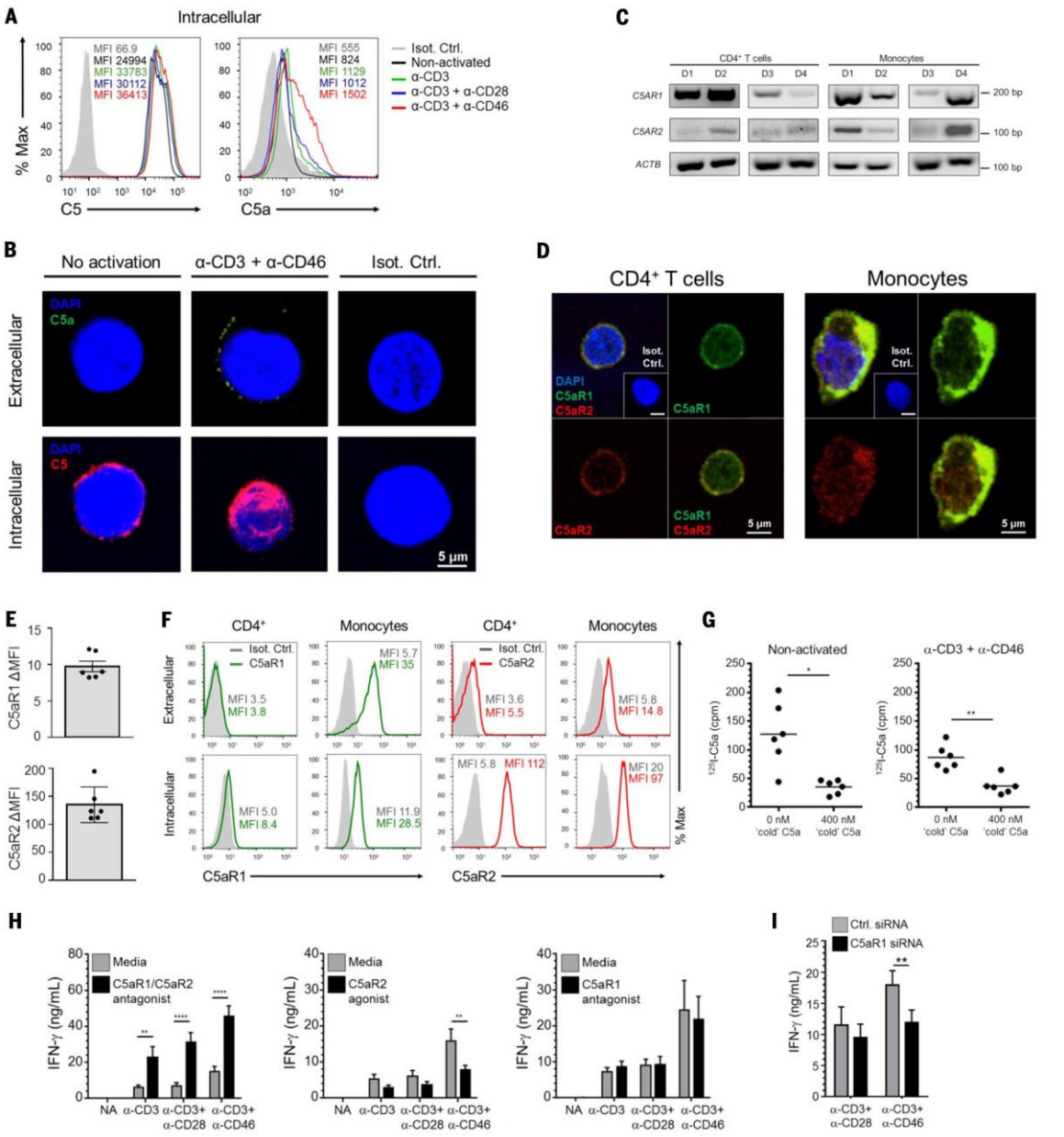

Fig. 1. Autocrine activation of C5a receptors regulates IFN-γ production by human CD4⁺ T cells. (**A** and **B**) Intracellular C5 and C5a generation in CD4⁺ T lymphocytes, left nonactivated or activated (36 hours) with anti-CD3 (α-CD3), α-CD3 + α-CD28, or α-CD3 + α-CD46 by flow cytometry (A) and confocal microscopy (B) (data representative of $n = 3$). (**C**) RT-PCR analysis for *C5AR1* and *C5AR2* mRNA in resting human CD4⁺ cells and monocytes ($n = 4$, donors D1 to D4, endogenous control *ACTB*). (**D**) Intracellular immunofluorescence on resting T cells and monocytes with antibodies to C5aR1 (green) and C5aR2 (red) (data are representative of $n = 3$). (**E**) C5aR1 and C5aR2 protein amounts in T cells with expression normalized to respective isotype control staining for each donor (change in mean fluorescence intensity ΔMFI ± SEM, $n = 6$). (**F**) Flow cytometry for C5aR1 and C5aR2 on resting T cells and monocytes, with representative histogram plots shown ($n = 6$). (**G**) Binding of radioactively labeled ^{125}I-C5a in absence or presence of nonlabeled "cold" C5a as competitor to resting or α-CD3 + α-CD46 activated (4 hours) T cells ($n = 6$). (**H**) IFN-γ secretion in nonactivated (NA) and activated (36 hours) CD4⁺ T cells in the absence or presence of a C5aR1/C5aR2 double receptor antagonist ($n = 9$), a C5aR2 agonist ($n = 8$), or a C5aR1 antagonist ($n = 7$). (**I**) IFN-γ production by T cells transfected with C5aR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) 36 hours after activation ($n = 7$). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. (G), paired *t* test; (H) and (I), two-way ANOVA with Bonferroni multiple comparison test.

immobilized antibodies to CD3, CD3 and CD28, or CD3 and CD46 in the presence or absence of (i) a specific antagonist to C5aR1 [PMX53 (*17*)]; (ii) the C5aR1/C5aR2 receptor double antagonist $A8^{\Delta 71\text{-}73}$ [dRA (*18*)], targeting only C5aR2 (as the C5aR1 is expressed intracellularly); or (iii) a specific C5aR2 agonist (*19*). All reagents were cell-impermeable. Blocking C5aR2 activity significantly increased T_H1 induction (Fig. 1H, left), and activating C5aR2 with the agonist or with C5a or C5adesArg reduced T_H1 responses (Fig. 1H, middle, and fig. S1E). Blockade of C5aR2 also led to increased T_H17 (IL-17) but not T_H2 (IL-4) responses (fig. S1F) without altering cell viability (fig. S1G). Consistent with the solely intracellular localization of C5aR1, the C5aR1-specific antagonist had no effect on IFN-γ production because it could not "reach" and block intracellular C5aR1 (Fig. 1H, right). However, reduction of intracellular C5aR1 by small interfering RNA (siRNA) gene targeting led to a commensurate decrease in IFN-γ production (Fig. 1I and fig. S1H). Together, these data show that intracellular C5 activation contributes to induction of IFN-γ production in $CD4^+$ T cells via intracellular C5aR1 engagement, and that the surface-expressed C5aR2 exerts negative control of IFN-γ, possibly via suppression of intracellular C5aR1 signals.

Canonical NLRP3 inflammasome activation in $CD4^+$ T cells enhances IFN-γ production

To delineate the autocrine C5-driven pathways contributing to regulation of IFN-γ in $CD4^+$ T cells, we performed a transcriptome analysis using T cells from three healthy donors activated, or not, with anti-CD3 and anti-CD46 in the presence or absence of the C5aR1/C5aR2 antagonist. Surprisingly, we observed enrichment of transcripts associated with inflammasome activation, including *NLRP3* and *IL1B* (Fig. 2, A and B, and table S1), in cells activated with anti-CD3 and anti-CD46. Inhibition of C5aR2 during these activation conditions further increased some of these transcripts, notably *IL1A* and *IL1B* (fig. S2A and table S2); this finding offers further support for the idea that blocking C5aR2 leads to unrestrained or increased engagement of intracellular C5aR1 driven by the anti-CD3– and anti-CD46–induced increase in intracellular C5a generation.

IL-1α and IL-1β are prototypical proinflammatory cytokines involved in innate immune responses and contributing to the development of several pathogenic autoimmune diseases, including

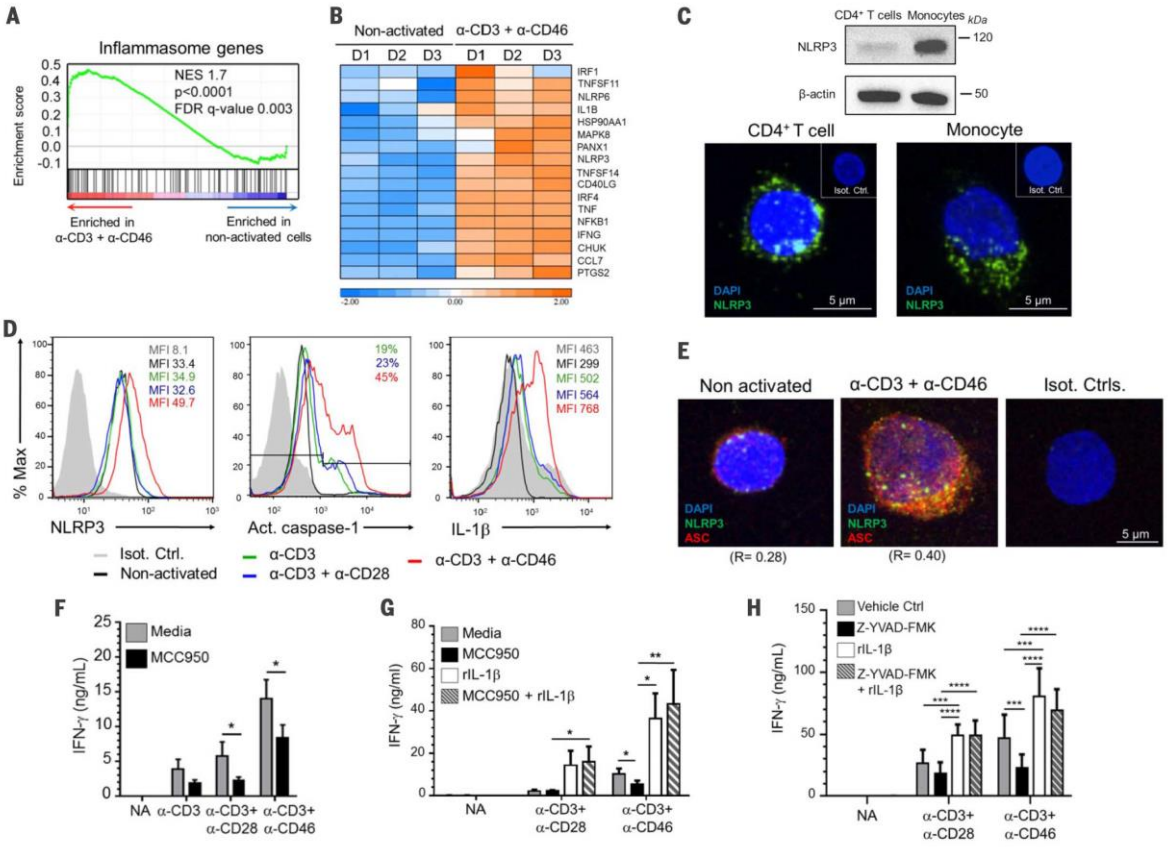

Fig. 2. NLRP3 inflammasome activation occurs in $CD4^+$ T cells and enhances IFN-γ production. (**A**) Gene set enrichment analysis (GSEA) for inflammasome-related genes in $CD4^+$ T cells after α-CD3 + α-CD46 activation (2 hours) compared to resting cells (donors D1 to D3). (**B**) Heat map depicting leading edge analysis (the core enriched genes) of the data in (A). (**C**) NLRP3 immunoblot (upper panel) and immunofluorescence (lower panel) on $CD4^+$ lymphocytes and monocytes (data representative of $n = 3$). (**D**) NLRP3, activated caspase-1 and total IL-1β protein expression in activated $CD4^+$ cells (data representative of $n = 3$). (**E**) Representative immunofluorescence costaining for NLRP3 (green) and ASC (red) on resting and α-CD3 + α-CD46 activated T cells (r = Pearson correlation coefficient between NLRP3 and ASC fluorescence, $n = 3$). (**F** and **G**) IFN-γ production by resting (NA) and activated $CD4^+$ T cells with or without MCC950 addition ($n = 7$) (F) and with or without rhIL-1β supplementation ($n = 3$) (G). (**H**) IFN-γ production in presence of the specific caspase-1 inhibitor Z-YVAD-FMK with or without rhIL-1β addition. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (F) to (H), two-way ANOVA with Bonferroni multiple comparison test.

type 1 diabetes and arthritis (*20*–*22*). Both IL-1α and IL-1β bind to IL-1 receptor 1 (IL-1R1). Antigen-presenting cell (APC)–derived IL-1β supports T cell priming and imprinting of T helper effector function (*23*), including enhancement of IFN-γ and IL-17 production from $CD4^+$ T cells (*24*–*26*). Further, mice with deletion of the IL-1β signal transducer MyD88 in T lymphocytes cannot generate memory T cells (*27*). Pro–IL-1β is synthesized as a 31-kDa precursor and converted to mature 17-kDa IL-1β via caspase-1 cleavage (*28*). Caspase-1 is regulated by proteolytic activation during oligomerization with NLRP3 and the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which is triggered in response to danger signals (*29*, *30*). NLRP3 inflammasome function requires a priming signal 1 (which induces *NLRP3* and *IL1B* gene transcription) and a signal 2 that induces functional inflammasome assembly (*30*) and has been described in myeloid innate immune cells, with monocytes as the main source of IL-1β (*25*, *31*), and in several nonimmune cell types (such as microglia, endothelial cells, and retinal pigment epithelial cells) (*32*–*34*). However, canonical NLRP3 inflammasome activity has not been demonstrated in lymphoid adaptive immune cells.

We confirmed the presence of an "NLRP3 signature" in T cells by demonstrating *NLRP3* and *IL1B* gene (fig. S2B) and protein expression, as well as generation of activated caspase-1 and mature IL-1β, in activated human $CD4^+$ T cells (Fig. 2, C and D, and fig. S2, C to F). Consistent with our gene array data, anti-CD3 and anti-CD46 activation led to robust NLRP3 activation and IL-1β generation (Fig. 2D) and increased colocalization of NLRP3 and ASC (Fig. 2E). Notably, both resting naïve and memory $CD4^+$ T cells expressed NLRP3 protein (fig. S2, C and D).

Because IL-1β supports T_H1 induction (*35*) and is most strongly induced by the T_H1 driver CD46, we next assessed whether inhibition of NLRP3 activity in $CD4^+$ T cells perturbs IFN-γ production. To this end, $CD4^+$ T cells were activated in the presence of MCC950, a specific NLRP3 inhibitor (*36*), and T_H1, T_H2, and T_H17 cytokine production was measured 36 hours after activation. NLRP3 inhibition during T cell activation specifically attenuated IFN-γ (Fig. 2F), whereas differences in IL-4 and IL-17 production did not reach significance (fig. S2G) and cell viability was unaffected (fig. S2H). The effects of the NLRP3 inhibitor could be fully reversed by the addition of recombinant human IL-1β (rhIL-1β) to cultures (Fig. 2G). Similarly, reduction of active caspase-1 activity by the specific inhibitor Z-YV AD-FMK repressed IL-1β and IFN-γ secretion (Fig. 2H and fig. S2I), and rhIL-1β provision normalized T_H1 induction in these cultures. The role for IL-1β as critical autocrine "T_H1 supporter" is reinforced by our observation that no IL-18 [which also depends on NLRP3 activation and can support T_H1 responses (*37*)] was measurable in our cultures and that addition of IL-18 binding protein had no effect on cytokine production (fig. S2J).

The hyperactive in vitro T_H1 response in CAPS patients is normalized by NLRP3 inhibition

To further explore this pathway, we measured the effects of NLRP3 hyperactivity in $CD4^+$ T cells isolated from the blood of patients with distinct gain-of-function mutations in NLRP3 (patient characteristics are summarized in table S3). This class of NLRP3 mutations is associated with a group of heritable monogenic syndromes known as cryopyrin-associated periodic syndromes (CAPS), characterized by excessive production of IL-1β from APCs with recurrent fevers, skin rashes, joint and ocular inflammation, and amyloidosis (*38*). Therapeutic suppression of the inflammatory responses can be achieved by IL-1R blockade with anakinra, an IL-1R antagonist, or canakinumab, a monoclonal antibody (mAb) targeting IL-1β (*38*, *39*). Despite their medication regimen and the fact that cytokine production by immune cells from CAPS patients can vary with their respective "flare status" (*40*), T cells from a first cohort of CAPS patients that we assessed had significantly increased IL-1β secretion relative to sex- and age-matched healthy donors (Fig. 3A), indicating that increased NLRP3 activity in $CD4^+$ T cells indeed induces heightened IL-1β secretion.

We next performed a more in-depth analysis of T cell in vitro responses from another cohort of seven CAPS patients (table S4). All patients had a naïve versus memory T cell distribution comparable to those of healthy donors (fig. S3A), and T cells from five patients of this second cohort also showed significantly increased IL-1β secretion upon activation (Fig. 3B). Furthermore, $CD4^+$ T cells from these patients trended toward substantially increased IFN-γ relative to T cells from sex- and age-matched healthy donors, and we observed a statistically significant correlation between increased IL-1β and IFN-γ secretion (Fig. 3, C and D).

T cells from CAPS patients displayed significantly reduced in vitro IL-17 responses (Fig. 3E).

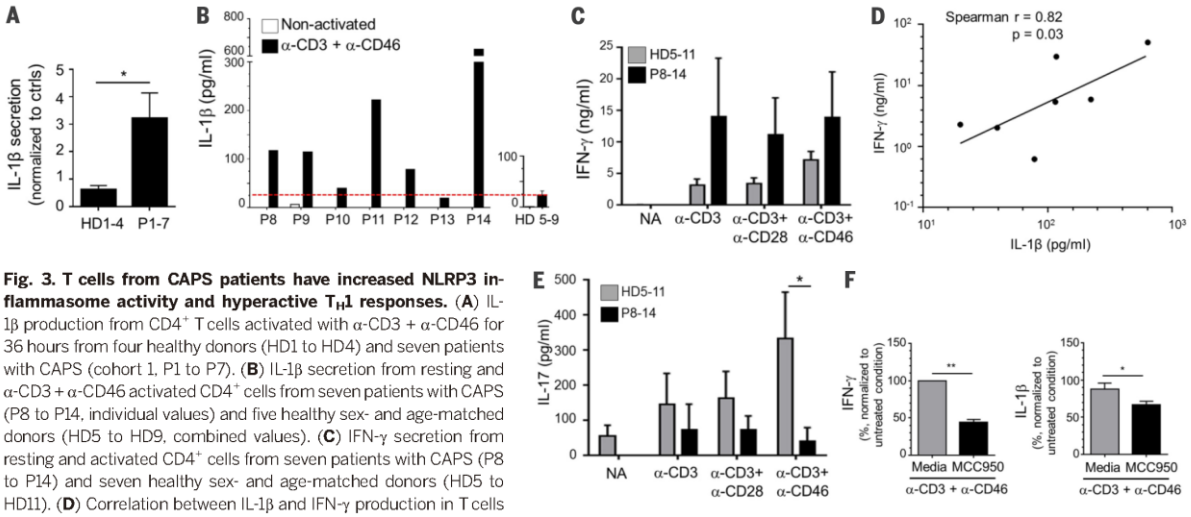

Fig. 3. T cells from CAPS patients have increased NLRP3 inflammasome activity and hyperactive T_H1 responses. (**A**) IL-1β production from $CD4^+$ T cells activated with α-CD3 + α-CD46 for 36 hours from four healthy donors (HD1 to HD4) and seven patients with CAPS (cohort 1, P1 to P7). (**B**) IL-1β secretion from resting and α-CD3 + α-CD46 activated $CD4^+$ cells from seven patients with CAPS (P8 to P14, individual values) and five healthy sex- and age-matched donors (HD5 to HD9, combined values). (**C**) IFN-γ secretion from resting and activated $CD4^+$ cells from seven patients with CAPS (P8 to P14) and seven healthy sex- and age-matched donors (HD5 to HD11). (**D**) Correlation between IL-1β and IFN-γ production in T cells from patients P8 to P14 upon α-CD3 + α-CD46 activation (Spearman correlation analysis). (**E**) IL-17 production by resting and activated T cells from CAPS patients P8 to P14 and healthy donors H5 to H11. (**F**) IFN-γ and IL-1β secretion by $CD4^+$ T cells from P8, P11, and P14 after α-CD3 + α-CD46 activation with or without MCC950 treatment (% normalized to nontreated). Analyses on (A) to (F) were performed at 36 hours after activation. Values correspond to two technical replicates for every patient and healthy control sample in each experiment. Data are means ± SEM. $*P < 0.05$, $**P < 0.01$. (A), unpaired *t* test; (C) and (E), two-way ANOVA with Bonferroni multiple comparison test; (D), Spearman correlation test; (F), paired *t* test.

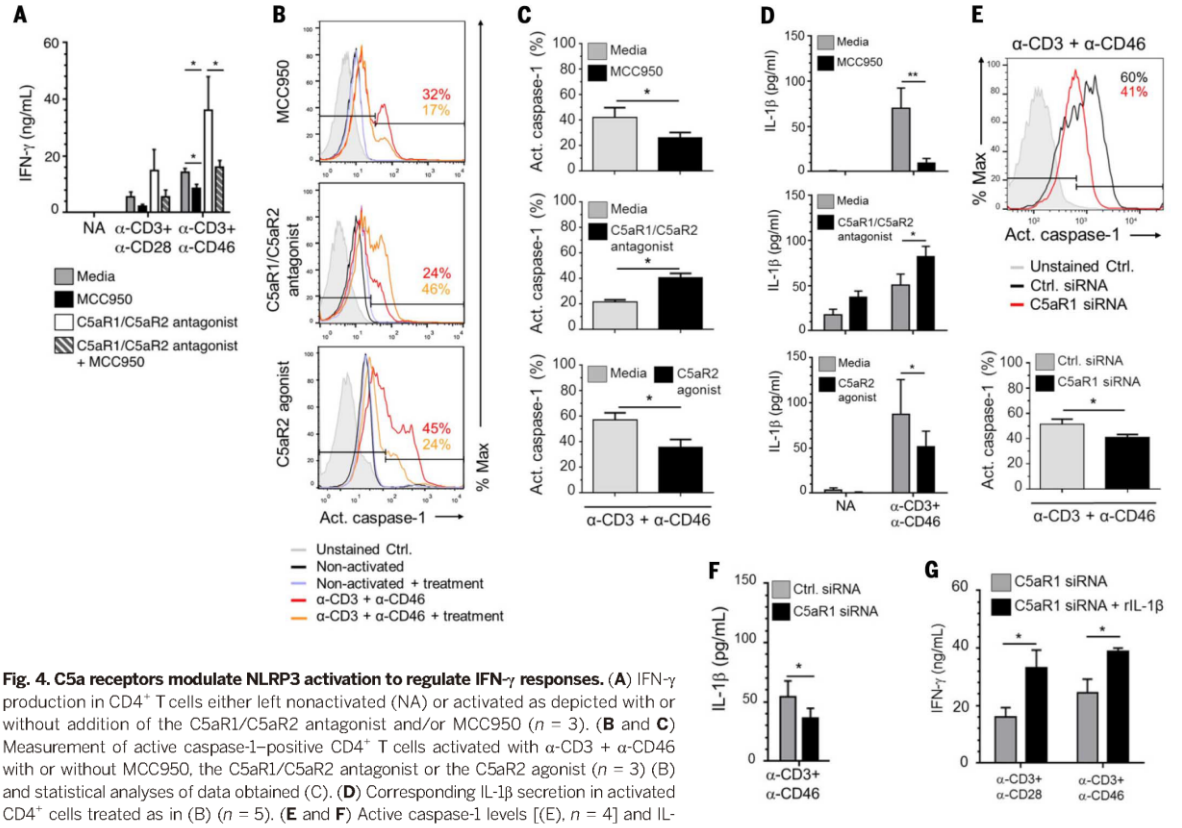

Fig. 4. C5a receptors modulate NLRP3 activation to regulate IFN-γ responses. (**A**) IFN-γ production in CD4$^+$ T cells either left nonactivated (NA) or activated as depicted with or without addition of the C5aR1/C5aR2 antagonist and/or MCC950 (n = 3). (**B** and **C**) Measurement of active caspase-1–positive CD4$^+$ T cells activated with α-CD3 + α-CD46 with or without MCC950, the C5aR1/C5aR2 antagonist or the C5aR2 agonist (n = 3) (B) and statistical analyses of data obtained (C). (**D**) Corresponding IL-1β secretion in activated CD4$^+$ cells treated as in (B) (n = 5). (**E** and **F**) Active caspase-1 levels [(E), n = 4] and IL-1β secretion [(F), n = 7] in T cells after transfection with either C5aR1-specific siRNA or scrambled control (Ctrl.) siRNA. (**G**) IFN-γ production in activated CD4$^+$ T cells after transfection with C5aR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) with or without addition of rhIL-1β (n = 3). Analyses were performed at 36 hours after activation. Data are means ± SEM. *P < 0.05, **P < 0.01. (A), (D), and (G), two-way ANOVA with Bonferroni multiple comparison test; (C), (E), and (F), paired t test.

Although caspase-1 activity was not significantly increased in the patients' T cells at the time point assessed (36 hours), the patients with highest IL-1β secretion also had the highest active caspase-1 levels (fig. S3B). Activation of CD4$^+$ T cells from CAPS patients in the presence of the NLRP3 inhibitor MCC950 led to a reduction of both IL-1β and IFN-γ secretion (Fig. 3F). Together, these data demonstrate that human CD4$^+$ T cells produce IL-1β in an NLRP3-dependent manner, that autocrine IL-1β generation supports IFN-γ secretion, and that dysregulation of this pathway occurs in human autoinflammatory disease.

C5a receptors modulate NLRP3 inflammasome activity to regulate IFN-γ production

We next asked whether C5aR signaling could directly regulate NLRP3 activity in human CD4$^+$ T cells. C5aR2 blockade in CD3 + CD46–activated T cells further increased *IL1B* but not *NLRP3* mRNA (fig. S4, A and B). Enhanced IFN-γ secretion driven by C5aR2 blockage could be reversed by inhibition of NLRP3 with MCC950 (Fig. 4A) without affecting IL-17 or IL-4 production (fig. S4C). Pharmacological targeting of C5aR2 via either dRA (blockage of C5aR2 signaling) or a C5aR2 agonist (activation of C5aR2 signaling) revealed that C5aR2 negatively regulates active caspase-1 and mature IL-1β expression in T cells (Fig. 4, B to D) but does not affect NLRP3 protein levels per se (fig. S4D). Silencing of *C5AR1* expression had also no effect on NLRP3 protein levels (fig. S4E) but reduced active caspase-1 (Fig. 4E) and IL-1β expression (Fig. 4F). Moreover, the reduction of IFN-γ secretion after *C5AR1* gene silencing was "rescued" by addition of rhIL-1β (Fig. 4G). Together, these data suggest that CD46-mediated signals increase *NLRP3* mRNA expression in T cells, whereas C5aR1 supports subsequent NLRP3 assembly and C5aR2 is a negative regulator of this process.

Reactive oxygen species (ROS) are "classical" upstream stimulators (signal 2) of NLRP3 assembly (*41*) and are strongly induced by C5aR1 in monocytes and neutrophils (*42*). Furthermore, generation of ROS within CD4$^+$ T cells is required for T cell activation and induction of IL-2, a key cytokine for T_H1 biology (*43*). We therefore assessed whether autocrine C5aR1 engagement by intracellular C5a generation in T cells induces NLRP3 inflammasome assembly via ROS generation. We observed potent generation of ROS in anti-CD3– and anti-CD46–induced T_H1 cells (Fig. 5A) and poor T_H1 induction in the presence of a ROS inhibitor (Fig. 5B). Further, reduction of C5aR1 protein expression by gene silencing decreased ROS production (Fig. 5C, left), whereas inhibition of C5aR2 surface activation significantly increased ROS generation in T cells (Fig. 4C, right).

Enhanced IFN-γ production by T cells induced by C5aR2 blockade could be entirely reversed by the presence of a ROS inhibitor (Fig. 5B and fig. S5). This finding suggests that NLRP3 activation in human T cells involves intracellular C5-driven ROS production.

NLRP3 activity in CD4$^+$ T cells is required for optimal IFN-γ responses during viral infection

To address the biological importance of NLRP3-driven autocrine IL-1β production by CD4$^+$ T cells, we analyzed CD4$^+$ T cell responses of *Nlrp3*$^{-/-}$,

Il1a$^{-/-}$/*Il1b*$^{-/-}$, and *Il1r1*$^{-/-}$ mice initially in vitro and subsequently in an established in vivo viral infection model. Similar to human CD4$^+$ lymphocytes, CD4$^+$ cells from wild-type mice expressed NLRP3 and IL-1β; neither NLRP3 nor IL-1β mRNA (Fig. 6A) and protein (fig. S6A) were detectable in T cells from respective gene-deficient animals. We observed no difference in the proportion of naïve versus memory T cells or in T cell survival between wild-type and knockout strains (fig. S6, B and C). However, upon in vitro CD3 + CD28 activation, CD4$^+$ T cells from *Nlrp3*$^{-/-}$, *Il1a*$^{-/-}$/*Il1b*$^{-/-}$, and *Il1r1*$^{-/-}$ mice had a reduction of ~75% in IFN-γ production when compared to T cells from wild-type animals (Fig. 6B); in contrast, IL-10, IL-4, and IL-17 production were unaffected in all three mouse mutant lines (fig. S6D). Further, although activation of T cells from wild-type mice in the presence of the NLRP3 inhibitor MCC950 had no effect on cell viability (fig. S6E), only IFN-γ production was significantly reduced (Fig. 6C and fig. S6F). These results indicate that diminished IFN-γ secretion in the "knockout T cells" was not due to a developmental defect, whereas NLRP3 activity is required for normal IFN-γ induction. Moreover, both naïve and memory mouse CD4$^+$ T cells displayed a requirement for NLRP3-driven IL-1β activity for optimal IFN-γ secretion (fig. S6, G and H).

Using a lymphocytic choriomeningitis virus (LCMV) model (Fig. 6D), we next demonstrated an in vivo role for NLRP3-driven IL-1β generation in T_H1 responses during infection. Irradiated mice were reconstituted with equal parts bone marrow cells isolated from wild-type mice mixed with bone marrow cells from *Nlrp3*$^{-/-}$, *Il1a*$^{-/-}$/*Il1b*$^{-/-}$, or *Il1r1*$^{-/-}$ mice before infection with LCMV. Analysis of splenic CD4$^+$ T cells 12 days after infection revealed comparable numbers of GP66-77$^+$Ki67$^+$ LCMV tetramer$^+$ cells generated by all animals (Fig. 6, E and F), indicating that *Nlrp3*$^{-/-}$, *Il1a*$^{-/-}$/*Il1b*$^{-/-}$, and *Il1r1*$^{-/-}$ CD4$^+$ T cells survived normally. However, T cells deficient in any of these components displayed substantially reduced ability to generate IFN-γ$^+$ virus-specific cells in vivo (with an average decrease of ~50%) (Fig. 6, G and H). Together, these data demonstrate that autocrine canonical NLRP3 inflammasome activity is required for optimal protective IFN-γ production by CD4$^+$ T cells during viral infection.

Autocrine NLRP3 activity in T cells controls the T_H1-T_H17 balance during intestinal inflammation

To further substantiate the in vivo importance of NLRP3 inflammasome activity in CD4$^+$ T cells, we also measured the effects of NLRP3 deficiency in an autoimmune disease setting by assessing its influence on disease outcome in a CD4$^+$ T cell transfer model of colitis, where IL-1β and both T_H1 and T_H17 responses in the intestine have been shown to be involved (*44*, *45*). To this end, sorted CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 wild-type or *Nlrp3*$^{-/-}$ mice were injected intraperitoneally (i.p.) into age- and sex-matched C57BL/10 *Rag2*$^{-/-}$ mice. Body weight and disease score were monitored and cytokine production by lamina propria CD4$^+$ T cells measured after animals displayed disease symptoms and were killed. Unexpectedly, relative to mice injected with wild-type CD4$^+$ T cells, mice that had received *Nlrp3*$^{-/-}$ CD4$^+$ T cells developed more severe disease with significantly increased weight loss, reduction in colon length, and higher disease scores (Fig. 7, A to C). Similar to our observation in the LCMV model, colonic *Nlrp3*$^{-/-}$ T cells displayed a substantial reduction in IFN-γ production (average decrease ~45%); however, we also observed a concurrent significant increase in T_H17 responses in these animals (Fig. 7, D and E). These observations were confirmed using a CD4$^+$ T cell–driven model of graft-versus-host disease (GvHD) where mice receiving *Nlrp3*$^{-/-}$ T cells also displayed more severe illness with reduced T_H1 but concurrently increased T_H17 induction (fig. S7, A to D). Together, these data demonstrate that the NLRP3 inflammasome mediates functionally important CD4$^+$ T cell intrinsic effects that not only are required for normal IFN-γ production but also control the T_H1-T_H17 balance during (at minimum) intestinal inflammation. These latter findings align with our observation that T cells from CAPS patients indeed have increased T_H1 but also decreased in vitro T_H17 responses (Fig. 3, C and E).

Discussion

Our results show that canonical NLRP3 inflammasome function is not confined to innate immune cells but is operative in adaptive CD4$^+$ T cells and, via autocrine IL-1β activity, is required for the optimal production of the key host defense factor IFN-γ. Further, and unexpectedly, NLRP3 assembly in human T cells requires TCR-induced intracellular C5 activation and stimulation of intracellular C5aR1, which initiates the generation of ROS and thereby provides a critical signal 2 for inflammasome assembly (*30*). Secretion of intracellularly generated C5a/C5ades-Arg engages the surface-expressed "alternative" C5aR2, which negatively controls NLRP3 activation either through inhibition of the C5aR1 or via a yet undefined mechanism. Given that APCs provide generally ample amounts of IL-1β during the cognate APC/T cell interaction, our observation that normal IFN-γ production requires also T cell autocrine IL-1β production is initially somewhat surprising. However, we envisage that, whereas APC-derived NLRP3-activated IL-1β supports initial T_H1 priming, proper "imprinting" or maintenance of the T_H1 phenotype during differentiation and migration into the periphery may rely on autocrine NLRP3 activity. IL-1β production by T cells, relative to myeloid cells, is comparatively low and, as we have shown, tightly regulated by an autocrine C5aR1 versus C5aR2 activation balance. The likely reason for this is that rapid control of local IL-1β is critical to normal termination of T_H1 responses: Human T_H1 cells co-induce IL-10 secretion in a CD46-dependent fashion during their contraction phase, and failure of this "IL-10 switch" underlies hyperactive T_H1 responses observed in rheumatoid arthritis and multiple sclerosis (*3*, *9*). IL-1β is a strong suppressor of IL-10 production (*23*) and, accordingly, we found that blockade of C5aR2 increased the IFN-γ/IL-10 ratio in CD4$^+$ T cells (fig. S8A), whereas

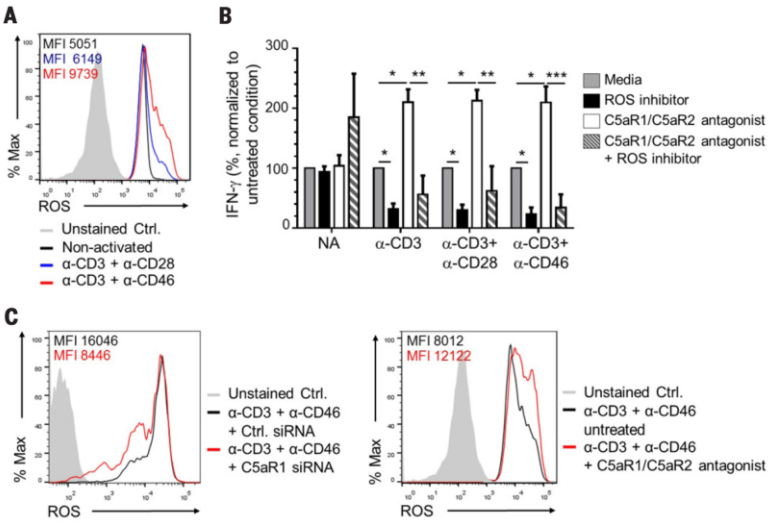

Fig. 5. Intracellular C5aR1 activation induces ROS generation in CD4$^+$ T cells. (**A**) ROS production in CD4$^+$ T cells activated under the depicted conditions (data shown are representative of $n = 3$). (**B**) IFN-γ production from CD4$^+$ T cells left nonactivated or activated as indicated with and without a specific ROS inhibitor and/or the C5aR1/C5aR2 antagonist ($n = 3$). Data are from a two-way ANOVA with Bonferroni multiple comparison test. (**C**) ROS production in α-CD3 + α-CD46 activated CD4$^+$ cells after transfection with C5aR1-specific siRNA (left panel) or with or without the C5aR1/C5aR2 double antagonist (right panel) (data shown are representative of $n = 3$). Analyses were performed 36 hours after activation. Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

IL-1β addition to cultures increased IFN-γ (Fig. 2G) but blocked proportional IL-10 secretion (fig. S8B). Moreover, IFN-γ to IL-10 switching was significantly reduced in T cells from CAPS patients (fig. S8C).

Further supporting the notion that autocrine T cell cytokine production needs to be carefully controlled in the microenvironment is our observation that the reduction in IFN-γ secretion by T cells from *Nlrp3*$^{-/-}$ mice led to a concurrent increase in colonic lamina propria T_H17 cells and increased intestinal inflammation. These findings demonstrate that T_H1 cells negatively control the influx and/or expansion of T_H17 cells during colitis and that T_H17 induction at this location (and in this model) is independent of intrinsic T cell NLRP3 activity. These observations may also help to explain why some groups observed protection of *Nlrp3*$^{-/-}$ animals in models of inflammatory bowel disease while others observed aggravated disease (*46*, *47*); these earlier studies had not controlled for a T cell–intrinsic function of the NLRP3 inflammasome. Also, because IL-1β also boosts the production of other cytokines including IL-4 and IL-17 (*35*), the activation of the NLRP3 inflammasome in T cells and its functional outcome could be context-dependent. For example, Bruchard *et al.* recently observed a noncanonical function for NLRP3 in mouse CD4$^+$ T cells (they did not assesses human T cells) independent of inflammasome formation and IL-1β secretion, during T_H2 induction and tumor growth (*48*).

In addition, there are clear species-specific differences in the relative contributions of complement receptor activities to IL-1β and/or IFN-γ induction in CD4$^+$ T cells. Although we found that CD4$^+$ T cells from *C5ar2*$^{-/-}$ mice have increased in vitro IFN-γ production, which was reduced to normal levels by MCC950 treatment (fig. S9), and *C5ar1*$^{-/-}$ mice have impaired in vitro and in vivo T_H1 responses (*49*), the role and expression of anaphylatoxin receptors on mouse T cells remains a matter of controversy (*49*–*51*). But more important, mice lack expression of CD46 on all immune cells (*6*), whereas in humans, CD46 costimulation is required for IL-2R assembly and the metabolic reprogramming in CD4$^+$ T cells that drives IFN-γ secretion (*5*, *7*, *52*). Further, we show here that CD46 engagement, aside from amplifying intracellular C5a generation and ROS production (signal 2), also delivers an important signal 1 for NLRP3 inflammasome activation by mediating increased transcription of the *NLRP3* gene [this likely occurs via CD46-induced increased NF-κB nuclear translocation during T cell activation (*6*)]. Because a functional homolog for CD46 has not been identified in rodents, the exact upstream signals controlling NLRP3 inflammasome activation and IL-1β production in murine T cells remain to be defined.

In summary, the regulated cross-talk between intracellularly activated complement components ("complosome") and the NLRP3 inflammasome emerges as fundamental to human T_H1 induction and regulation. That established innate immune pathways previously not thought to be operative

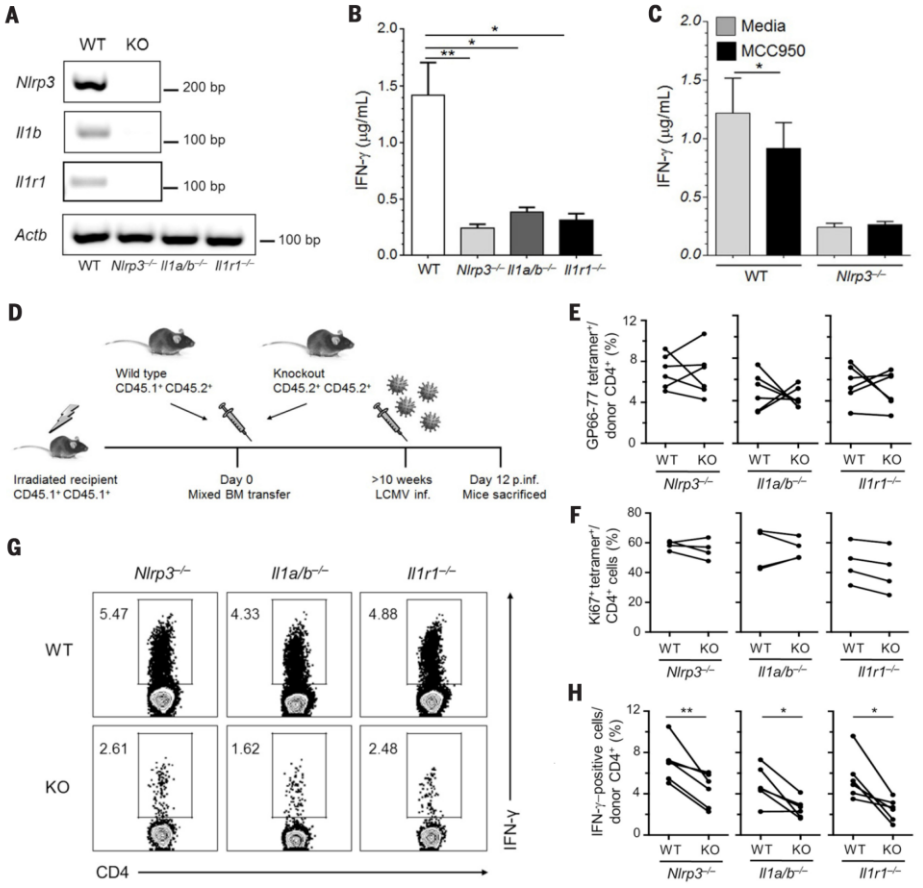

Fig. 6. NLRP3 function in CD4$^+$ T cells drives optimal IFN-γ production during viral infection. (**A**) RT-PCR analysis on CD4$^+$ T cells isolated from wild type (WT), *Nlrp3*$^-$, combined *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ (*Il1a/b*$^{-/-}$) and *Il1r1*$^{-/-}$ mice for corresponding gene mRNA expression. (**B**) Cytokine secretion from CD4$^+$ T cells isolated from wild-type and knockout mice at 96 hours after α-CD3 + α-CD28 activation (n = 3). (**C**) Cytokine production from CD4$^+$ T cells from wild-type and *Nlrp3*$^{-/-}$ mice after α-CD3 + α-CD28 activation (96 hours) with or without addition of MCC950 (n = 4). (**D**) Schematic of the acute lymphocytic choriomeningitis virus (LCMV) infection model used in this study. (**E** and **F**) Percentage of LCMV tetramer-positive CD4$^+$ T cells isolated from the spleens of the three bone marrow chimeric mice groups used 12 days after infection (E) and percentages of Ki67$^+$GP66-77$^+$/tetramer-positive cells (F). (**G** and **H**) Representative intracellular IFN-γ staining in splenic CD4$^+$ T cells of one mouse from each group after LCMV peptide restimulation (5 hours) [(G), n = 6] with corresponding statistical analyses [(H), n = 6]. Data are means ± SEM. *P < 0.05, **P < 0.01. (B), one-way ANOVA with Tukey multiple comparison test; (C), two-way ANOVA with Bonferroni multiple comparison test; (E) to (H), paired t test. Data in (G) are representative of two independent experiments; data in (E), (F), and (H) are pooled from two independent experiments.

in adaptive immune cells are not only present but also are key in directing immunological responses is of substantial importance to our understanding of immunobiology and immune system evolution. Further, the possibility that this normal functional cross-talk may also be target of viral immune evasion strategies (*53*) suggests that the complement-NLRP3 axis in T cells could represent a novel therapeutic target for the modulation of IFN-γ responses in autoimmunity and infection. In this regard, it will be valuable to explore whether optimal production of IFN-γ by $CD8^+$ T cells (*54*), natural killer T (NKT) cells, and/or innate lymphoid type 1 (ILC1) cells also relies on autocrine complement-NLRP3 inflammasome activity.

Materials and methods

Healthy donors and patients

Blood samples were obtained with ethical and institutional approvals (Wandsworth Research Ethics Committee, REC number 09/H0803/154). T cells were purified blood samples from healthy volunteers after informed consent. Fourteen adult patients with CAPS were recruited at the National Amyloidosis Centre, University College London (ethical approval REC reference number 06/Q0501/42) with key information on the patients summarized in tables S3 and S4. In all experiments that involved T cells from CAPS patients, T cells from age- and sex-matched healthy volunteers were used as controls.

Mice used in the study

All mice used in this study are on a C57BL/6 background (with the exception of the GvHD experiment, where Balb/c mice were used). Wild-type and *Il1r1*$^{-/-}$mice were purchased from Jackson Laboratories and subsequently backcrossed to B6 for 10 generations at NIH (Bar Harbor, ME). The *C5ar2*$^{-/-}$ (human gene symbol GPR77) mice were previously described (*12*), *Nlrp3*$^{-/-}$ animals were provided by V. Dixit of Genentech, and mice deficient in *Il1a* and *Il1b* (*Il1a/Il1b*$^{-/-}$ animals) were kindly provided by Y. Iwakura (Tokyo University) (*55*). The C57BL/10 $RAG2^{-/-}$ mice were obtained from Taconic. All animals were maintained in AALAC-accredited BSL2 or BSL3 facilities at the NIH or FDA and experiments performed in compliance with an animal study proposal approved by the NIAID or FDA Animal Care and Use Committee.

Cell isolation and activation

Human cells: $CD4^+$ T cells and monocytes were isolated from blood as previously published using the MACS Human $CD4^+$ Positive T cell Isolation Kit or the MACS Human $CD14^+$ Cell Positive Isolation Kit (both Miltenyi Biotech, Bisley, UK), respectively (*7*). Purity of bead-isolated T lymphocyte fractions was typically >98% and for monocytes >95%. For naïve and memory $CD4^+$ T cell sorting, cells were stained with appropriate antibodies (naïve cells, $CD4^+$, $CD45RA^+$, $CD45RO^-$, and $CD25^-$; memory cells, $CD4^+$, $CD45RA^-$, $CD45RO^+$, and $CD25^-$) and sorted with a BD FACSAria II Cell Sorter (KCL Flow Core facility). $CD4^+$ T cells were activated in 48-well culture plates (2.5×10^5 to 3.0×10^5 cells per well) coated with mAbs to CD3, CD28, or CD46 (2.0 μg/ml PBS each) and addition of rhIL-2 (25 U/ml), thus, under nonskewing conditions. Monocytes were activated in 24-well plates (2.5×10^5 to 5.0×10^5 cells per well) by addition of LPS (50 ng/ml). Cell viability was monitored by either propidium iodide (BD Biosciences) or the LIVE/DEAD Cell Viability Assay (Life Technologies).

Mouse cells: Single cell suspensions of spleen cells were generated and red blood cells lysed using ACK lysis buffer (Life Technologies). $CD4^+$ T cells were isolated by negative selection using the Stem Cell Technologies EasySep Mouse $CD4^+$ T Cell Isolation Kit (Tukwila, WA). To obtain pure $CD4^+$ T cell populations, $CD4^+$ cells were sorted using a FACS Aria (BD Biosciences) based on $CD4^+$ $CD45.2^+$ staining and to separate naïve versus memory $CD4^+$ T lymphocytes. T cells were sort-separated based on $CD4^+$ $CD44^+$ (memory) and $CD4^+$ $CD44^-$ (naïve) stainings. For in vitro T cell activation, 48- or 96-well plates were coated with anti-CD3 (2 μg/ml) overnight at 4°C, and $CD4^+$ T cells (0.5×10^6 to 1.0×10^6 per well of 48-well plates or 0.2×10^6 per well of 96-well plates) were added to the appropriate wells. Anti-CD28 (1 μg/ml) was added to the media to provide costimulation.

Lymphocytic choriomeningitis virus (LCMV) infection in mice

Preparation of mixed bone marrow (BM) chimeric mice: B6.SJL (CD45.1,1) mice were lethally irradiated (950 rad) and reconstituted with a total of 10^7 donor BM cells from C57BL/6 CD45.1,2 wild-type mice mixed at equal parts with BM cells from CD45.2,2 mice deficient (KO) in *Nlrp3*$^{-/-}$, *Il1r1*$^{-/-}$,

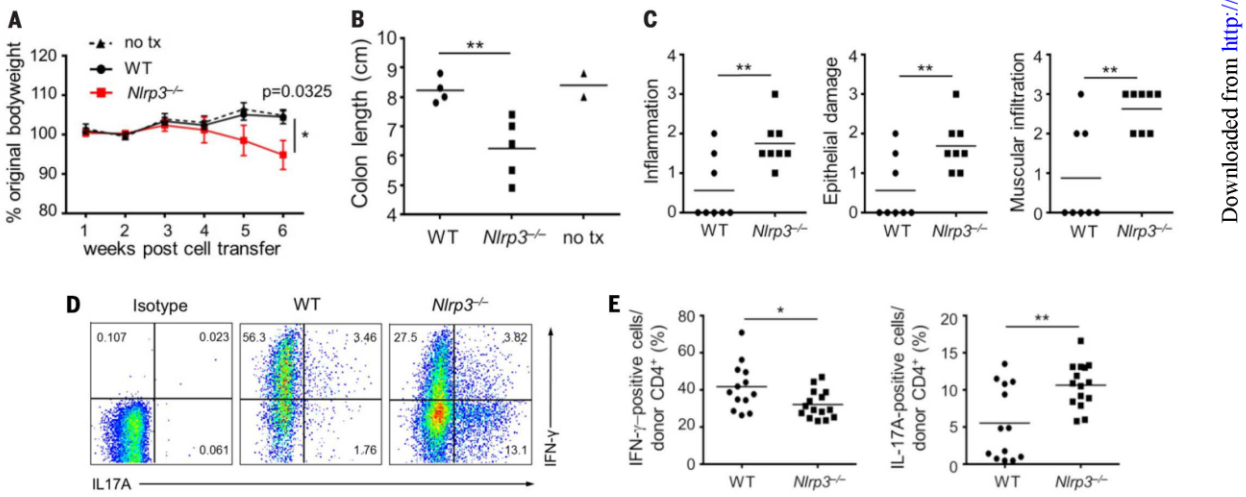

Fig. 7. T cell intrinsic NLRP3 activity regulates the T_H1-T_H17 balance in intestinal inflammation. (**A** to **E**) Naïve splenic $CD25^-CD45RB^{hi}$ $CD4^+$ T cells from wild-type or *Nlpr3*$^{-/-}$ mice were transferred into C57BL/10 *Rag2*$^{-/-}$ mice. (**A**) Weight change over the course of colitis induction. (**B**) Colon length at the study endpoint. (**C**) Inflammation score of the colons according to blinded histological analysis with assessment of inflammation (left panel), epithelial damage (middle panel) and muscular immune cell infiltration (right panel). (**D** and **E**) Intracellular IFN-γ and IL-17A staining of colonic $CD4^+$ T cells at the study endpoint after overnight α-CD3 + α-CD28 stimulation and brefeldin A and monensin addition for 5 hours (gated on live $CD4^+$ $Thy1.2^+$ T cells). Representative flow cytometric plots (D) with corresponding statistical analysis shown from two combined independent experiments [(E), $n = 13$ wild-type, $n = 15$ KO]. Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$. (A) and (B), one-way ANOVA with Sidak multiple-comparisons test; (C) and (E), unpaired t test. Data are representative of two experiments [(A) to (C)] or are combined from two experiments (E).

or *Il1a/Il1b*$^{-/-}$. Mice were allowed to reconstitute for 10 weeks before infection with LCMV.

LCMV infection and assessment of antigen-specific CD4$^+$ T cell response: Ten weeks after reconstitution, the mice were infected i.p. with 10^5 pfu of LCMV-Armstrong. On day 12 after infection, the mice were killed and the spleens removed for processing. For ex vivo cytokine staining of mouse cells after LCMV infection, cells were incubated with LCMV GP61-80 peptide (1 μg/ml) in the presence of monensin and brefeldin for 5 hours at 37°C. Staining for LCMV-specific CD4$^+$ T cells was performed using an APC-labeled $1A^b$ LCMV GP66-77 tetramer (NIH tetramer core facility) as described (*56*). Data were acquired with a FACS Calibur, Fortessa LSRIII, or FACS Aria cytometer (BD Biosciences) and analyzed with FlowJo 10.0.8 software (Ashland, OR).

Induction of colitis and colon cell isolation

Splenic CD4$^+$ T cells were isolated from C57BL/6 or *Nlpr3*$^{-/-}$ mice using a negative selection CD4 T cell enrichment kit (Stemcell tech), were stained with anti-CD45RB FITC, anti-CD25 APC, and anti-CD4 BV421, and sorted on a FACS Aria (BD biosciences) for CD4$^+$ CD25$^-$CD45RB^{hi} (brightest 35%) cells. Wild-type or *Nlpr3*$^{-/-}$ cells (2×10^5 each) were injected i.p. into age- and sex-matched C57BL/10 RAG2$^{-/-}$ mice. The mice were killed when symptoms of clinical disease (5 to 10% weight loss of original body weight and/or diarrhea) were observed in at least one group, approximately 6 to 11 weeks after adoptive transfer. Colon lamina propria cells were isolated as described (*57*), with the additional step of further purifying the cells over a 44 and 67% Percoll gradient to enrich for the mononuclear cells.

Scoring of intestinal inflammation

Samples of the proximal, mid-, and distal colon were excised after feces were flushed from the colons, placed into 3.7% formaldehyde solution, and then paraffin-embedded. Cross-sectional sections were cut and stained with hematoxylin and eosin (H&E). Colon pathology scores were based on severity of mononuclear cell inflammation, intestinal wall thickening, including infiltration to the muscularis, and epithelial damage, including edema, degeneration, and necrosis on a graded scale where 0 = normal, 0.5 = very mild, 1 = mild, 2 = moderate, 3 = severe. Samples were scored blinded by a pathologist from the NIH Pathology Score.

Induction of graft versus host disease (GvHD)

Balb/c mice were lethally irradiated with 900 cGy (two doses of 450 cGy 3 hours apart) on day −1. C57BL/6 wild-type bone marrow was depleted of T cells with the use of a CD90.2 Positive Selection Kit (Stemcell tech), and 5×10^6 cells were transferred on the following day (day 0) alone (control), or in addition to 1×10^6 wild-type B6 or *Nlpr3*$^{-/-}$ naïve CD4$^+$ T cells isolated with the Negative Selection Naïve CD4 T Cell Kit (Stemcell tech). Mice were killed upon clinical symptoms of disease (diarrhea and weight loss) on day 12 after cell transfer.

Detection of active caspase-1 and reactive oxygen species (ROS)

Generation of cleaved and active caspase-1 in cells was monitored by Western blotting for appropriate active fragment generation and by using the Green FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer's protocol with subsequent FACS analysis. ROS staining was performed by incubating cells to be assayed in dihydrorhodamine 123 (17 μg/ml) diluted in Hank's balanced salt solution with 10 mM HEPES (all from Sigma Aldrich) for 15 min at 37°C. Data were acquired on a FACS Calibur or Fortessa LSRIII cytometer (BD Biosciences) and analyzed with FlowJo software.

Confocal microscopy

Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained with the indicated primary antibodies overnight and with secondary antibodies for 30 min at 4°C. Cells were mounted using VECTASHIELD media with DAPI (Vector Laboratories, Burlingame, CA) and images were acquired with a Nikon AIR confocal microscope (Nikon Imaging Centre, King's College London) and analyzed using NIS Elements (Nikon) and ImageJ software (National Institutes of Health).

Binding studies with recombinant human ^{125}I-labeled C5a

CD4$^+$ T cells from healthy donors were left nonactivated or activated for 4 hours with immobilized antibodies to CD3 and CD46 and then incubated for 2 hours at 4°C (1×10^7 cells/ml) with 10 μl of 0.1 nM ^{125}I-rhC5a (Perkin Elmer) and either 400 nM nonlabeled rhC5a in HAG-CM buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% bovine serum albumin, 0.5 mM glucose, pH 7.4) or buffer without rhC5a addition. Cells were vacuum-transferred onto 96-well MultiScreen-HV filter plates (MAHVN4510; Millipore/Merck), nonbound ^{125}I-rhC5a removed by washing and cell-bound ^{125}I-rhC5a detected on the filter membranes by ^{125}I using a Packard Cobra II Gamma Counter (Perkin Elmer). For binding controls, HEK 293 cells (ATCC CRL 1573) were stably transfected with the pQCXIN vector expressing hC5aR1 or hC5aR2 (leading to expression of >1 Mio. of the respective C5aR/cell) or with the "empty" vector as control (*58*) (these cell lines also served as specificity controls for the anti-C5a receptor antibodies used in this study). In order to get comparable low CPM values as observed with purified T cells, only 5×10^4 cells/ml of C5aR1- or C5aR2-expressing HEK cells were applied. They were diluted in buffer containing "no-C5aR-expressing control" cells. The constant higher number of cells (5×10^5 HEK cells/ml in the 30-μl volume later used in the binding assay) permitted repetitive washing without cell loss and ensured identical nonspecific binding in all samples containing the same cell type. C5aR1-, C5aR2-expressing or control HEK 293 cells were incubated for 1 hour with or without 100 nM of nonlabeled rhC5a, washed thoroughly and then incubated for an additional 2 hours with 10 μl of 0.1 nM ^{125}I-rhC5a. After removal of nonbound rhC5a, binding of ^{125}I-C5a to the respective HEK 293 cell lines was determined by measuring gamma radioactivity. To exclude C5a-induced C5aR-internalization during all binding studies all steps in the binding experiments were performed at 4°C and HEK 293 cells were additionally preincubated 15 min at 37°C with 0.1% NaAcid and Cytochalasin B (21 μg/ml) and then cooled on ice for 5 min before their incubation with rhC5a.

Cytokine measurements

Cytokine production by cells in culture was quantified from cell supernatants using either the human or mouse $T_H1/T_H2/T_H17$ Cytometric Bead Array (BD Bioscience) or via intracellular cytokine staining after treated for 4 hours with PMA (50 ng/ml), ionomycin (1 μg/ml) (both Sigma Aldrich), and 1× Golgi Plug (BD Biosciences). Secreted human IL-1β and IL-18 were measured using the Human IL-1β/IL-1F2 DuoSet Kit or the Human IL-18 Platinum ELISA kit (R&D Systems and eBiosciences, respectively) in combination with SIGMAFAST OPD tablets (Sigma Aldrich) as substrate for detection.

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and quantitative RT-PCR

RNA was extracted with the RNeasy Mini Kit including DNase digestion and DNA cleanup (Qiagen), and reverse transcription was performed with the One Step RT-PCR (Qiagen). For quantitative PCR, RNA was reverse-transcribed with 2.5 μM random hexamers, 1 mM dNTPs, 40 U of RiboLock RNase inhibitor, and 400 U of RevertAid H Minus Reverse Transcriptase (Thermo Scientific). Quantitative PCR was performed using KI-Q Hot Start Sybr Green Mix (Sigma Aldrich), with 150 pmol of forward and reverse primers and data acquired on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in table S5.

RNA silencing

SiRNA targeting human C5AR1 mRNA and control scrambled siRNA were purchased from Origene (Rockville, MD) and delivered at a final concentration of 15 nM (mixture of three different C5aR1 siRNA used at 5 nM each or scramble control at 15 nM) into primary human CD4$^+$ T cells by transfection with Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's instructions. *C5AR1* mRNA level reduction was consistently about 30%.

Microarray data generation and analysis

Transcriptome profiling was performed by the KCL Genomic Centre (London) using human exon 1.0 ST arrays (Affymetrix) on CD4$^+$ T cells isolated from three different healthy donors that were left either nonactivated or were activated with antibodies to CD3 and CD46 for 2 hours in the absence or presence of the C5aR1/C5aR2 antagonist. Expression data were analyzed using

Partek Genomics Suite (Partek Inc., St. Louis, MO) version 6.6 and Gene Set Enrichment Analysis, GSEA (*59*) (Broad Institute of MIT and Harvard) with a normalized enrichment score of 1.8 to derive normalized enrichment score (NES), nominal *P* value, and FDR *q* value. Microarray data sets were used in conjunction with the Qiagen-generated inflammasome gene set (84 members). Heat maps for the leading edge subset were drawn with Partek genomics suite. Table S1 shows the normalized read values from microarrays for Fig. 2, A and B. The list of annotated genes differentially regulated by the C5aR1/C5aR2 double antagonist (fig. S2) is given in table S2.

Statistical analysis

Analyses were performed with GraphPad Prism (La Jolla, CA). Data are presented as mean ± SEM and compared using either paired *t* tests with Bonferroni correction for multiple comparisons, one- or two-way analysis of variance (ANOVA) with a Tukey multiple comparison post hoc test or with Sidak multiple-comparisons test, as appropriate. Correlation analysis (Fig. 3D and fig. S3B) was performed with Spearman's correlation test. *P* values < 0.05 denote statistical significance.

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SUPPLEMENTARY MATERIALS

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2.4 Research article supplement (Arbore *et al*., Science, 2016)

Supplementary Materials for

T helper 1 immunity requires complement-driven, NLRP3 inflammasome activity in $CD4^+$ T cells

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This PDF file includes:

Additional Materials and Methods (list of antibodies, proteins, agonists and antagonists)
Figs. S1 to S9
Tables S1 to S5

Materials and Methods

Antibodies, proteins, agonists and antagonists

Cell-stimulating mAbs to human $CD4^+$ T cells were bought from BD Biosciences, San Diego, CA (anti-hCD28, CD28.2), purified from a specific hybridoma (anti-hCD3; OKT-3) or generated in-house (anti-CD46; TRA-2-10) (*3*). Mouse T cells were activated with anti-CD3 (145-2C11) and anti-CD28 (37.51) from Bio X Cell (West Lebanon, NH). The anti-human/mouse NLRP3 (ab4207), anti-human C5 (ab66850) and C5a (ab11878), anti-human/mouse IL1-β (ab9722) and anti-human β-actin (ab8226) antibodies were purchased from Abcam (Cambridge, UK). The anti-human C5a antibody was also biotinylated in house using the APEX™ Biotin-XX Antibody Labeling Kit (Life Technologies Ltd, Paisley, UK). Alternative antibodies to human/mouse NLRP3/NALP3 (Clone 768319) and human/mouse IL-1β (3A6, used for Western blotting and FACS experiments) were purchased from R&D Systems (Minneapolis, MN) and Cell Signalling Technology (Beverly, MA), respectively. Additional antibodies used include anti-human NLRP3/NALP3 (AG-20B-0014-C100) and ASC (AL177) from Adipogen (Liestal, Switerland), anti-hC5aR1 (sc-53795) and anti-hNLRP3 (sc-34408) from Santa Cruz (Dallas, TX), anti-hC5aR2 (ID9-M12) and anti-hCD45RA from Biolegend, anti-hIL-1β (12-7018-81) and anti-hCD4 from eBioscience (San Diego, CA), anti-hC5aR1 (MCA2059; AbD Serotec, Oxford, UK), anti-hC5aR2 (PA1-41397; Thermo Scientific (Leicestershire, UK)), and anti-hcaspase-1 (3019-100; Biovision (Milpitas, CA)). The antibodies recognizing anti-human-CD25, CD45RA (555488), and CD45RO (559865) were purchased from BD Biosciences. The following mouse antibodies were purchased from Biolegend: Mouse Trustain (Fc-Block), anti-CD44 FITC, PE-cy7 or BV421, anti-mouse IFN-γ PE, anti-mouse CD4 (APC-cy7, BV 421 or BV605), anti-mouse CD45.1 FITC and CD45.2 PerCP, anti-mouse CD45RB FITC, anti-mouse CD25 APC. Anti-mouse/human Ki67 was purchased from BD Biosciences. The secondary antibodies anti-rabbit IgG H+L chain Alexa Fluor 594 (ab150076), anti-goat IgG H+L chain PE (ab7004) and anti-goat IgG H+L chain Alexa Fluor 488 (ab150129) were from Abcam, while anti-mouse IgG Alexa Fluor 488 (A11001), anti-rabbit IgG H+L chain Alexa Fluor 594 (A11037) and anti-rat IgG H+L chain Alexa Fluor 488 (A11006) were obtained from Molecular Probes/Life Sciences (Paisley, UK). APC streptavidin was purchased from Biolegend.

Recombinant active human IL-1β was bought from Abcam and used at 20 ng/ml in cultures, recombinant human IL18BPa (Sino Biologicals Inc.) was used at 50 μM, human IL-2 was provided by C. Pham (Washington University in Saint Louis, MO) and lipopolysaccharide (LPS) was purchased from Sigma Aldrich (Saint Louis, MO). Recombinant C5 and C5adesArg were purchased from CompTech (Tyler, TX). The specific C5aR1 antagonist (PMX53) (*17*) was provided by T. Woodruff (University of Queensland, AU) and used at 10 μM, the C5aR1/C5aR2 double antagonist a gift from J. Köhl (University of Lübeck, Germany) (*18*) and used at 7 μM, and the specific C5aR2 agonist (RHYPYWR) was generated by T. Woodruff and P. Monk (Sheffield University, UK) (*19*) and used at 100 μM. The specific NLRP3 inhibitor MCC950 (*36*) was used at 10 μM, the specific caspase-1 inhibitor Z-YVAD-FMK (Abcam) was used at 20 μM and the reactive oxygen species (ROS) inhibitor Diphenyleneiodonium (DPI) (Sigma Aldrich) was added at 750 nM. In all experiments, cells were incubated in media for 15 minutes including the compound

of choice (incubation with the corresponding vehicle buffer was used as control) before activation and culture.

Figure legends

Suppl. Fig. 1. Autocrine activation of C5a receptors regulates IFN-γ production by human CD4$^+$ T cells. (A) C5aR1 and C5aR2 Western blot analyses on cytoplasmic (Cyt.) and membrane (Mem.) fractions of resting human CD4$^+$ cells (representative of n=3). **(B** and **C)** Representative flow cytometry histograms for intracellular staining **(B)** and immunoblot with cytoplasmic (Cyt.) and membrane (Mem.) fractions **(C)** on C5aR1 and C5aR2 in HEK293 cells (HEK) transfected with a vector expressing either C5aR1, or C5aR2, or an empty control vector (Ctrl. Vec.). **(D)** Binding of radioactively-labelled ^{125}I-C5a to HEK293 cells expressing either C5aR1, C5aR2 or no C5a receptor in the absence or presence of non-labelled 'cold' C5a as competitor (n=3). **(E)** IFN-γ production in CD4$^+$ T cells activated for 36 hours with α-CD3 + α-CD46 in presence of increasing concentrations of exogenous C5a or C5adesArg (n=3), with significance assessed between untreated cells (0 ng/mL C5a or C5adesArg) and cells treated with indicated amounts of either C5a or C5adesArg. **(F)** IL-17 and IL-4 production by non-activated (NA) and activated (36 hours) CD4$^+$ T cells in the absence or presence of either a C5aR1/C5aR2 double receptor antagonist (n=9), a C5aR2 agonist (n=8) or a C5aR1 antagonist (n=7). **(G)** Cell viability of T cells either resting or activated for 36 hours as indicated in the absence or presence of the C5aR1/C5aR2 double antagonist or C5aR2 agonist (n=2). **(H)** Reduction of *C5AR1* mRNA levels in T cells transfected with either a C5AR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) 48 hours post transfection. Left panel shows a representative mRNA expression sample and the right sample statistically significant reduction in *C5AR1* mRNA expression in C5AR1-specific siRNA-treated CD4$^+$ T cells (n=6). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 (D to G, two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test; H, paired *t*-test).

Suppl. Fig. 2. NLRP3 inflammasome activation occurs in CD4$^+$ T cells and enhances IFN-γ production. (A) Volcano plot showing transcripts differentially regulated in CD4$^+$ T cells from 3 donors after α-CD3 + α-CD46 activation (2 hours) with or without addition of the C5aR1/C5aR2 antagonist to cultures. **(B)** Quantitative RT-PCR to measure *NLRP3* and *IL1B* mRNA in non-activated (NA) or α-CD3 + α-CD46 activated human CD4$^+$ T cells at 36 hours post activation (n=3, expression normalized to *ACTB*). (**C** and **D**) Representative NLRP3 expression assessed by flow cytometry (**C**) and by immunofluorescence (**D**) in non-activated naïve and memory human CD4$^+$ T cells (n=3). **(E** and **F)** Representative caspase-1 and IL-1β immunoblot analyses (with lower arrows depicting the activated protein forms), performed on resting and α-CD3 + α-CD46 activated CD4$^+$ T cells (36 hours) and resting and LPS activated monocytes (50 ng/ml, 18 hours) (representative of n=4) with densitometric analyses on activated caspase-1 and IL-1β in T cells. The corresponding quantitative data shown below the immunoblots do not depict absolute amounts of proteins in monocytes versus T cells. They depict the ratio (percentage) of non-cleaved (non-activated) versus cleaved (activated) protein in either T cells or in monocytes. **(G)** IL-17 and IL-4 secretion in CD4$^+$ cells non-activated (NA) or activated as indicated with or without the NLRP3 inhibitor MCC950 at 36 hours post activation (n=7). **(H)** Cell viability of CD4$^+$ cells either resting or activated for 36 hours as indicated in the absence or presence of the NLRP3-specific inhibitor MCC950 (n=2). **(I)** IL-1β secretion by resting (NA) and activated CD4$^+$ T cells (36 hours) with or without addition of the caspase-1 inhibitor Z-YVAD-FMK (n=4). (**J**) IL-18 production in α-CD3 + α-CD46 activated CD4$^+$ T cells (36 hours) from three HDs (left panel) and IFN-γ production in CD4$^+$ T cells from HDs 1 and 2 by T cells activated with α-CD3 + α-CD46 for 36 hours in full media and for 72 hours in serum free media, in the presence of 50 μM of rIL18BP (right panel). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01 (E and F, paired *t*-test; G to I, two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

Suppl. Fig. 3. T cells from CAPS patients have increased caspase-1 activity and IL-1β secretion upon *in vitro* stimulation. (A) Percentages of naïve and memory CD4$^+$ T cell subpopulations in the blood of a second cohort of CAPS patients P8 to

P14 and of three sex- and age-matched healthy donors (HD5-HD7). **(B)** Correlation between active caspase-1 and IL-1β production in T cells from patients P8-P14 upon CD3 + CD46 activation (Spearman's correlation analysis).

Suppl. Fig. 4. C5a receptors regulate NLRP3 activation to modulate IFN-γ responses. (**A** and **B**) Quantitative RT-PCR to measure *IL1B* **(A)** and *NLRP3* mRNA **(B)** in resting or α-CD3 + α-CD46 activated (2 hours) human $CD4^+$ T cells in the absence or presence of the C5aR1/C5aR2 antagonist with the respective corresponding bar graphs (panel below) showing relative expression in activated versus non-activated cells with or without C5aR1/C5aR2 antagonist-treated T cells (n=3, expression normalized on *ACTB*). (**C**) IL-17 and IL-4 production in resting or activated T cells in presence or absence of MCC950 and/or the C5aR1/C5aR2 double antagonist at 36 hours (n=4). (**D**) NLRP3 expression in $CD4^+$ T lymphocytes either left non-activated or activated with α-CD3, α-CD3 + α-CD28 or α-CD3 + α-CD46 for 36 hours with or without addition of the C5aR1/C5aR2 antagonist (upper row) or the C5aR2 agonist (lower row) to cultures (data representative of n=3). **(E)** NLRP3 expression after α-CD3 + α-CD46 activation (36 hours) in T cells transfected with either C5aR1-specific siRNA or scrambled control (Ctrl.) siRNA (data representative of n=3). Error bar graphs represent mean ± SEM. *p <0.05 (A and B, paired *t*-test; C, two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

Suppl. Fig. 5. Effect of ROS inhibition on IL-17 and IL-4 production by $CD4^+$ T cells. IL-17 and IL-4 secretion (shown as % normalized to respective untreated conditions) from $CD4^+$ T cells left non-activated (NA) or activated as indicated with or without a specific ROS inhibitor and/or the C5aR1/C5aR2 antagonist at 36 hours post activation (n=3). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 (two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

Suppl. Fig. 6. NLRP3 function in $CD4^+$ T cells is required for optimal IFN-γ response *in vivo*. (**A**) Representative immunofluorescence analysis for NLRP3 and IL-1β protein expression on $CD4^+$ T cells isolated from wild type (WT), *Nlrp3*$^{-/-}$ and

combined *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ (*Il1a/b*$^{-/-}$) mice. **(B)** Percentages of naïve and memory CD4^{+} T cells isolated from the spleen of unchallenged wild type (WT) and *Nlrp3*$^{-/-}$, combined *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ (*Il1a/b*$^{-/-}$), and *Il1r1*$^{-/-}$ mice (n=3). **(C)** Cell viability of sorted CD4^{+} T cells from WT and knock out mice 96 hours post CD3 + CD28 activation (n=3). **(D)** IL-10, IL-4 and IL-17 secretion from CD4^{+} T cells isolated from WT and knock out mice activated 96 hours with antibodies to CD3 and CD28 (n=3). **(E)** Cell viability of sorted CD4^{+} T cells from WT and *Nlrp3*$^{-/-}$ mice assessed with or without MCC950 addition (right panel) during activation (96 hours post α-CD3 + α-CD28 activation, n=4). (**F**) IL-10, IL-4 and IL-17 secretion from CD4^{+} T cells activated 96 hours with antibodies to CD3 and CD28 from WT and *Nlrp3*$^{-/-}$ mice with or without addition of MCC950 (n=4). **(G and H)** IFN-γ, IL-10, IL-4 and IL-17 secretion from sorted naïve **(G)** and memory **(H)** CD4^{+} T cells from WT, *Nlrp3*$^{-/-}$, *Il1a/b*$^{-/-}$, and *Il1r1*$^{-/-}$ mice 96 hours post α-CD3 + α-CD28 activation (n=3). Error bar graphs represent mean ± SEM. **p* <0.05 (B, E and F, two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test; C, D, G and H, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test).

Suppl. Fig. 7. Lack of intrinsic NLRP3 inflammasome function in CD4^{+} T cells impacts on GvHD disease. (**A-D**) Disease scores and Th1 and Th17 T cell populations. T cell-depleted C57BL/6 bone marrow was transferred into lethally irradiated BALB/c mice alone (control group), or with the addition of 1 x 10^{6} naïve CD4^{+} T cell from either C57BL/6 or *Nlpr3*$^{-/-}$ mice. (**A**) Colon length at study endpoint (12 days post-cell transfer). (**B-D**) Intracellular IFN-γ and IL-17A staining of mesenteric lymph node CD4^{+} T cells at the study endpoint after overnight α-CD3 and α-CD28 stimulation and brefeldin A and monensin addition for 5 hours (Gated on live CD4^{+} Thy1.2^{+} C57BL/6 (H-2K^{d}D^{d+}) T cells). Percent (**B**) and mean fluorescence intensity (MFI) of IFN-γ^{+} cells (**C**) and percent IL-17A^{+} cells (**D**). For (**A-D**) n=4 WT, 5 KO, 2 controls. Error bar graphs represent mean ± SEM. **p* <0.05, ***p* <0.01, ****p* <0.001 (B to D, one-way ANOVA with Sidak's multiple comparisons test, A; unpaired *t*-test).

Suppl. Fig. 8. The C5aR2-NLRP3-IL-1β axis may regulate 'IL-10 switching' in human Th1 cells. (A) IFN-γ to IL-10 ratio in α-CD3 + α-CD28 or α-CD3 + α-CD46 activated (36 hours) $CD4^+$ T cells in the absence or presence of either a C5aR1/C5aR2 double receptor antagonist (n=9), a C5aR2 agonist (n=8) or a C5aR1 antagonist (n=7). **(B)** IL-10 production by resting and activated $CD4^+$ T cells, in presence or absence of MCC950 and/or rhIL-1β measured at 36 hours post activation (n=3). **(C)** IL-10 secretion at 36 hours post indicated activation by $CD4^+$ cells from the patients with CAPS P8-P14 and seven sex- and age-matched healthy donors (HD5-11) with data represented as mean ± SEM. **p* <0.05, ***p* <0.01 (two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

Suppl. Fig. 9. Cytokine production of T cells from *C5ar2*$^{-/-}$ mice in the presence and absence of NLRP3 inhibition. *In vitro* cytokine production of $CD4^+$ T cells from wild type and *C5ar2*$^{-/-}$ mice with or without addition of MCC950 at 48 hours post α-CD3 + α-CD28 activation. Error bar graphs represent mean ± SEM. **p* <0.05, ***p* <0.01 (two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

Table S1.

Normalized read values from microarrays for Fig. 2A and B
Table is provided in Other Supplementary Material as an Excel file.

ID	p-value	Fold change
C15orf48	0.0118739	2.08331
C3orf14	0.0327513	2.13952
CXCL2	0.0330927	5.59906
DDX60	0.00208813	2.38253
DTX3L	0.0101958	2.02692
EIF2AK2	0.00659247	3.45509
EREG	0.00093681	2.50735
FAM45B	0.035722	2.16729
HERC6	0.00197424	2.8311
HSH2D	0.0219705	2.13182
IFI44	0.00790994	2.89407
IFI44L	0.0204978	4.5496
IFIH1	0.00489751	2.63568
IFIT1	0.0250406	3.36767
IFIT3	0.0192085	4.57257
IFIT5	0.00982865	2.10946
IL1A	0.0137544	3.52362
IL1B	0.0053077	2.36807
IL6	0.0145825	3.11426
IL8	0.00751255	3.61565
LAMP3	0.0046064	2.25455
MX1	0.032786	4.21644
NM_001199779 // PSMB2	0.0148295	-2.09297
OAS2	0.010885	4.16812
OR4N2	0.0348681	-2.69263
PARP9	0.0314253	3.3435
PTGS2	0.00566655	3.80875
SAMD9	0.0116602	2.07468
SAMD9L	0.0364514	3.18497
SERPINB2	0.0268882	3.68178
TNFAIP6	0.0045844	2.61021

Table S2.

Genes differentially regulated by the C5aR1/C5aR2 double antagonist
Reported the p-value and the fold change (α-CD3 + α-CD46 + C5aR1/C5aR2 double antagonist vs α-CD3 + α-CD46)

Patient N°	Age/gender	NLRP3 Mutation	Treatment
1	59 y./female	V198M	Canakinumab
2	29 y./female	V198M	Canakinumab
3	45 y./male	T436I	Anakinra
4	53 y./male	mut neg (mosaic c.1699G>A, E567K, in 5.2% of cells)	Anakinra
5	72 y./male	A439V	Canakinumab
6	22 y./male	R260W	Canakinumab
7	16 y./male	R260W	Canakinumab

Table S3.

Details of seven patients (1 to 7) with cryopyrin-associated periodic syndrome (CAPS)

Patient N°	Age/gender	NLRP3 Mutation	Treatment
8	31 y./female	A439V	Canakinumab
9	52 y./female	A439V	Canakinumab
10	29 y./female	A439V	Canakinumab
11	55 y./female	A439V	Canakinumab
12	27 y./female	A439V	Starting canakinumab
13	60 y./female	A439V	Starting canakinumab
14	67 y./female	somatic mosaicism (none in germline)	On anakinra for 3 weeks

Table S4.

Details of seven patients (8 to 14) with cryopyrin-associated periodic syndrome (CAPS) and A439V mutation

Gene	Primer Forward	Primer Reverse
C5AR1	5'-CACTAGGGCCCAGAAGAC-3’	5'-AAGAGTCCCGCTGGAAAAGG-3’
C5AR2	5’-GCTCTTCCTGTATTTTGGGAGGG-3’	5’-GCTGGTGGATTTCTTGCTGTCC-3’
NLRP3	5’-GGCAACACTCTCGGAGACAAG-3’	5’-GCTCTGGCTGGAGGTCAGAA-3’
IL1B	5'-CTCGCCAGTGAAATGATGGCT-3'	5'-GTCGGAGATTCGTAGCTGGAT-3'
ACTB	5'-ACGGCCAGGTCATCACCATTG-3'	5'-AGTTTCGTGGATGCCACAGGAC-3'
Nlrp3	5’-AGCCAGAGTGGAATGACACG-3’	5’-CGTGTAGCGACTGTTGAGGT-3’
Il1b	5’-ATCAACCAACAAGTGATATTCTCCAT-3’	5’-GGGTGTGCCGTCTTTCATTAC-3’
Il1r1	5’-ATGGAAGTCTTGTGTGCCCT-3’	5’-TCCGAAGAAGCTCACGTTGT-3’
Actb	5’-GATGCCCTGAGGCTCTTTTCC-3’	5’-GAGGTCTTTACGGATGTCAACGTCA-3’

Table S5.
Listed primers sequences

Fig. S1

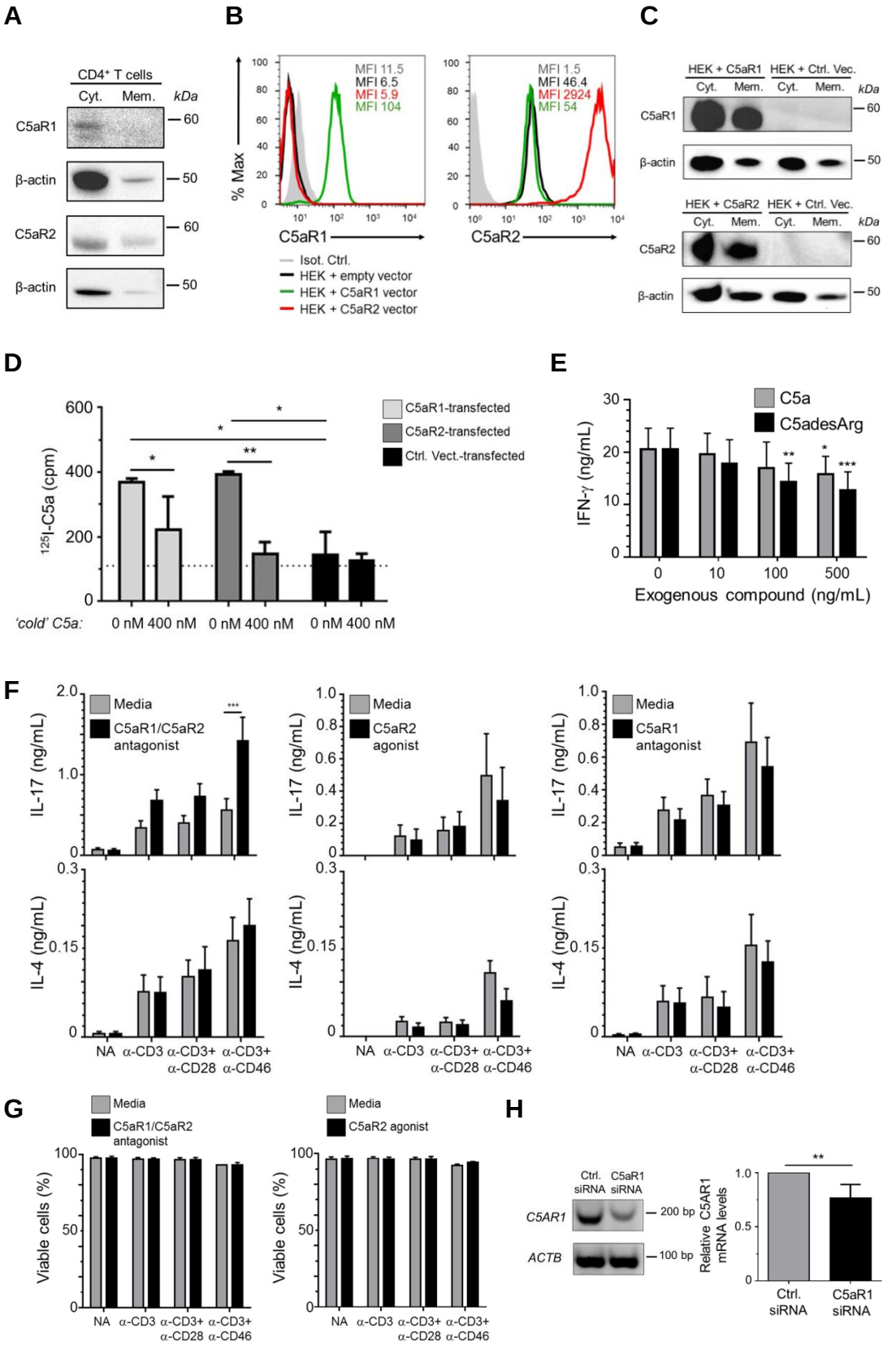

A
CD4⁺ T cells
Cyt.
Mem.
kDa
C5aR1
β-actin
C5aR2
β-actin
60
50
60
50
B
% Max
MFI 11.5
MFI 6.5
MFI 5.9
MFI 104
C5aR1
MFI 1.5
MFI 46.4
MFI 2924
MFI 54
C5aR2
Isot. Ctrl.
HEK + empty vector
HEK + C5aR1 vector
HEK + C5aR2 vector
C
HEK + C5aR1
HEK + Ctrl. Vec.
HEK + C5aR2
D
125I-C5a (cpm)
C5aR1-transfected
C5aR2-transfected
Ctrl. Vect.-transfected
'cold' C5a:
0 nM 400 nM 0 nM 400 nM 0 nM 400 nM
E
IFN-γ (ng/mL)
C5a
C5adesArg
Exogenous compound (ng/mL)
F
IL-17 (ng/mL)
IL-4 (ng/mL)
Media
C5aR1/C5aR2 antagonist
C5aR2 agonist
C5aR1 antagonist
NA
α-CD3
α-CD3+ α-CD28
α-CD3+ α-CD46
G
Viable cells (%)
C5aR1/C5aR2 antagonist
C5aR2 agonist
H
Ctrl. siRNA
C5aR1 siRNA
C5AR1
ACTB
200 bp
100 bp
Relative C5AR1 mRNA levels

Fig. S2

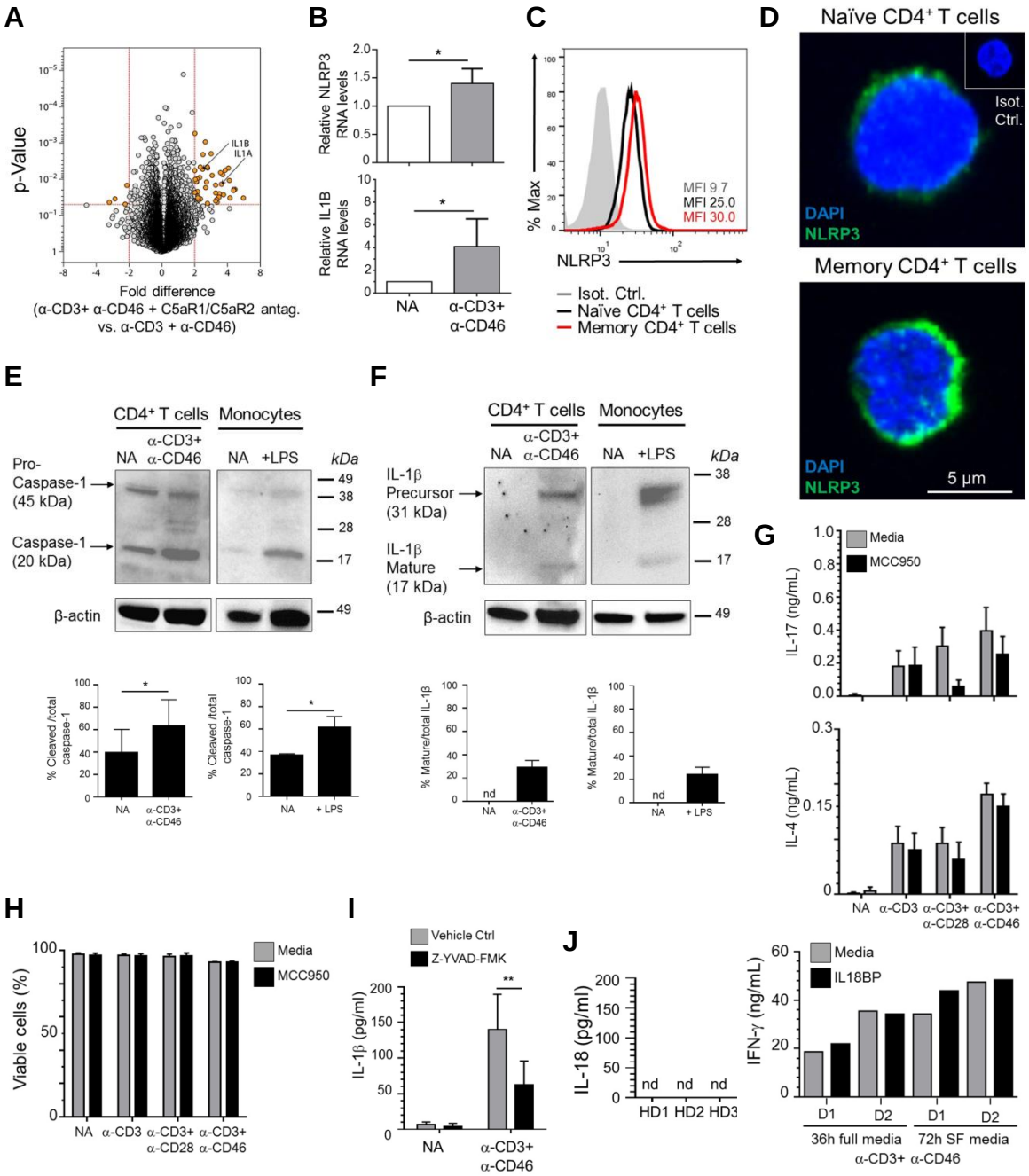

A
p-Value
IL1B
IL1A
Fold difference
(α-CD3+ α-CD46 + C5aR1/C5aR2 antag.
vs. α-CD3 + α-CD46)
B
Relative NLRP3 RNA levels
Relative IL1B RNA levels
NA
α-CD3+ α-CD46
C
% Max
MFI 9.7
MFI 25.0
MFI 30.0
NLRP3
Isot. Ctrl.
Naïve CD4+ T cells
Memory CD4+ T cells
D
Naïve CD4+ T cells
Isot. Ctrl.
DAPI
NLRP3
Memory CD4+ T cells
DAPI
NLRP3
5 µm
E
CD4+ T cells
Monocytes
α-CD3+ α-CD46
NA
+LPS
kDa
Pro-Caspase-1 (45 kDa)
Caspase-1 (20 kDa)
β-actin
% Cleaved /total caspase-1
+ LPS
F
IL-1β Precursor (31 kDa)
IL-1β Mature (17 kDa)
% Mature/total IL-1β
nd
G
Media
MCC950
IL-17 (ng/mL)
IL-4 (ng/mL)
NA
α-CD3
α-CD3+ α-CD28
α-CD3+ α-CD46
H
Viable cells (%)
I
Vehicle Ctrl
Z-YVAD-FMK
IL-1β (pg/ml)
J
IL-18 (pg/ml)
HD1 HD2 HD3
IFN-γ (ng/mL)
IL18BP
D1
D2
36h full media
72h SF media
α-CD3+ α-CD46

Fig. S3

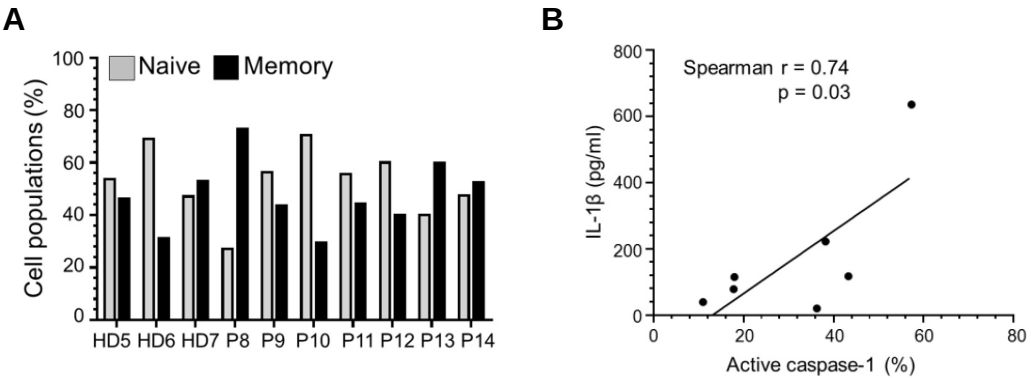

A
Naive
Memory
Cell populations (%)
100
80
60
40
20
0
HD5 HD6 HD7 P8 P9 P10 P11 P12 P13 P14
B
Spearman r = 0.74
p = 0.03
IL-1β (pg/ml)
800
600
400
200
0
0
20
40
60
80
Active caspase-1 (%)

Fig. S4

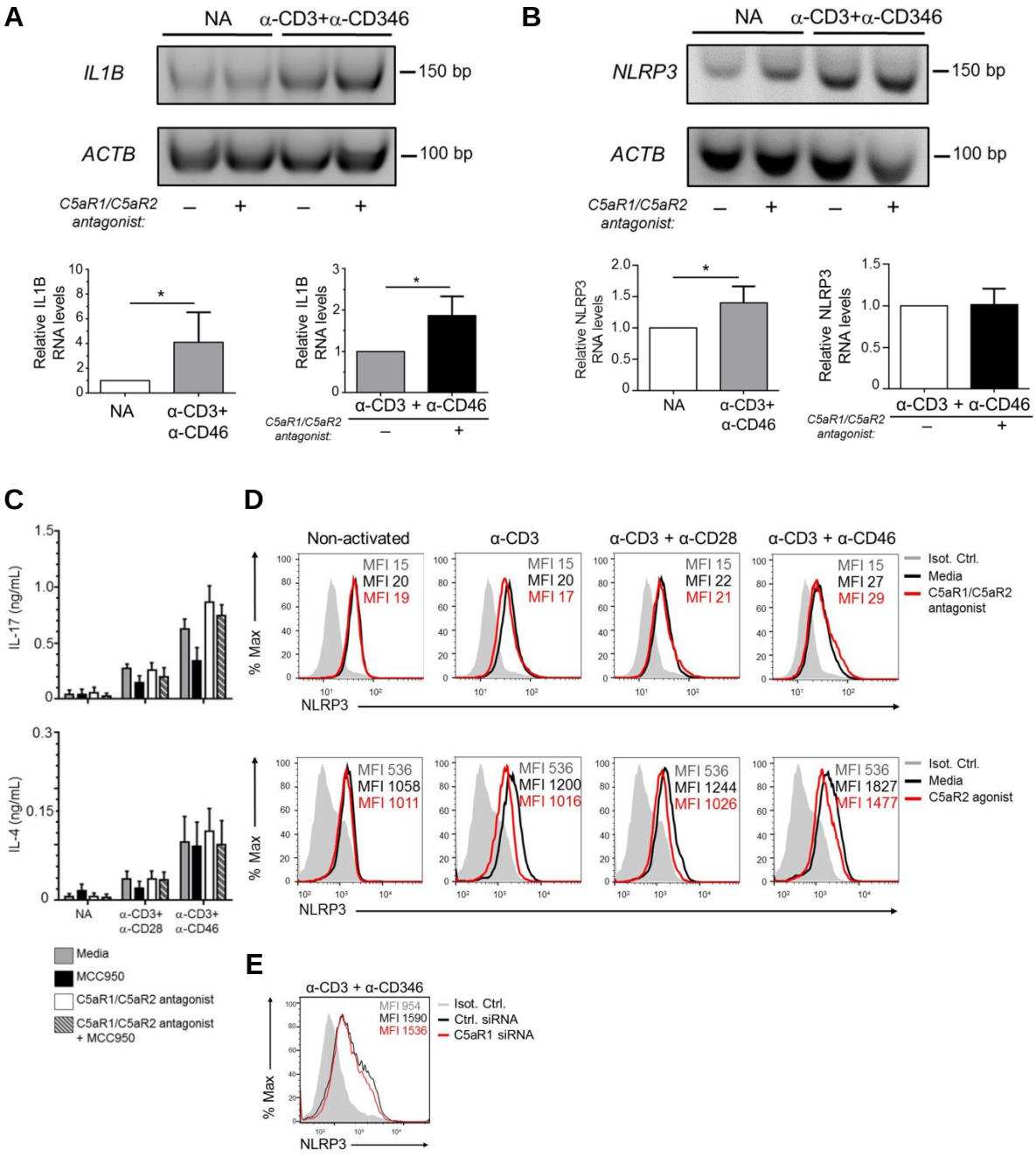

A
NA
α-CD3+α-CD346
IL1B
150 bp
ACTB
100 bp
C5aR1/C5aR2 antagonist:
Relative IL1B RNA levels
NA
α-CD3+ α-CD46
α-CD3 + α-CD46
B
NLRP3
Relative NLRP3 RNA levels
C
IL-17 (ng/mL)
IL-4 (ng/mL)
NA
α-CD3+ α-CD28
α-CD3+ α-CD46
Media
MCC950
C5aR1/C5aR2 antagonist
C5aR1/C5aR2 antagonist + MCC950
D
Non-activated
α-CD3
α-CD3 + α-CD28
α-CD3 + α-CD46
% Max
NLRP3
MFI 15
MFI 20
MFI 19
MFI 17
MFI 22
MFI 21
MFI 27
MFI 29
Isot. Ctrl.
Media
C5aR1/C5aR2 antagonist
MFI 536
MFI 1058
MFI 1011
MFI 1200
MFI 1016
MFI 1244
MFI 1026
MFI 1827
MFI 1477
C5aR2 agonist
E
α-CD3 + α-CD346
MFI 954
MFI 1590
MFI 1536
Ctrl. siRNA
C5aR1 siRNA

Fig. S5

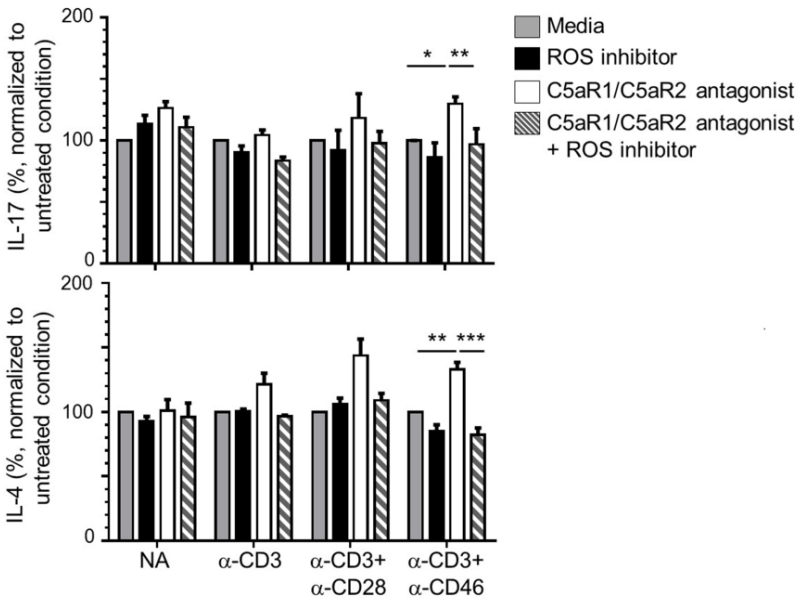

IL-17 (%, normalized to untreated condition)
IL-4 (%, normalized to untreated condition)
200
100
0
* **
** ***
Media
ROS inhibitor
C5aR1/C5aR2 antagonist
C5aR1/C5aR2 antagonist + ROS inhibitor
NA
α-CD3
α-CD3+ α-CD28
α-CD3+ α-CD46

Fig. S6

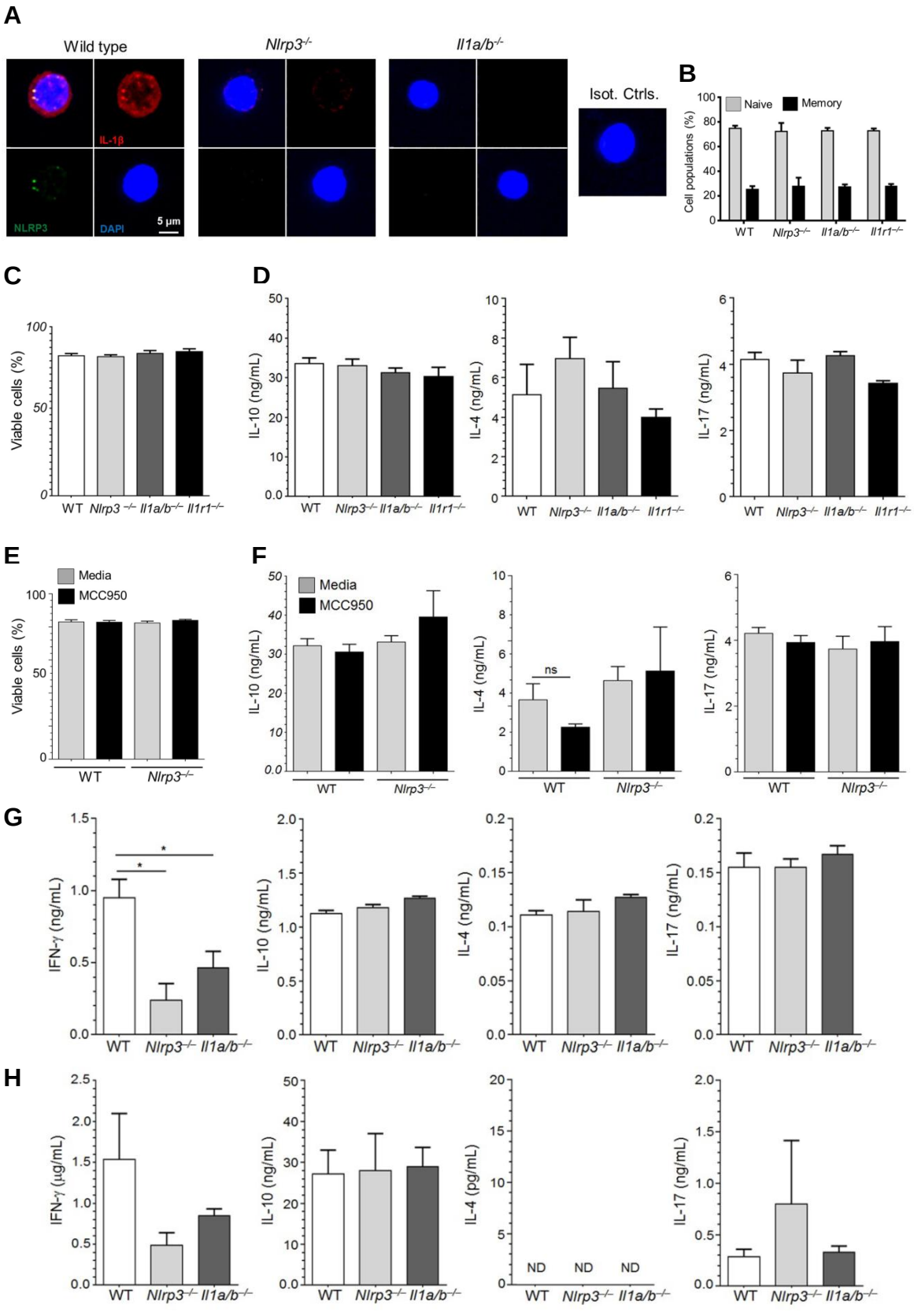

A
Wild type
Nlrp3-/-
Il1a/b-/-
Isot. Ctrls.
IL-1β
NLRP3
DAPI
5 µm
B
Naive
Memory
Cell populations (%)
WT
Nlrp3-/-
Il1a/b-/-
Il1r1-/-
C
Viable cells (%)
D
IL-10 (ng/mL)
IL-4 (ng/mL)
IL-17 (ng/mL)
E
Media
MCC950
F
ns
G
IFN-γ (ng/mL)
*
H
IFN-γ (µg/mL)
ND

Fig. S7

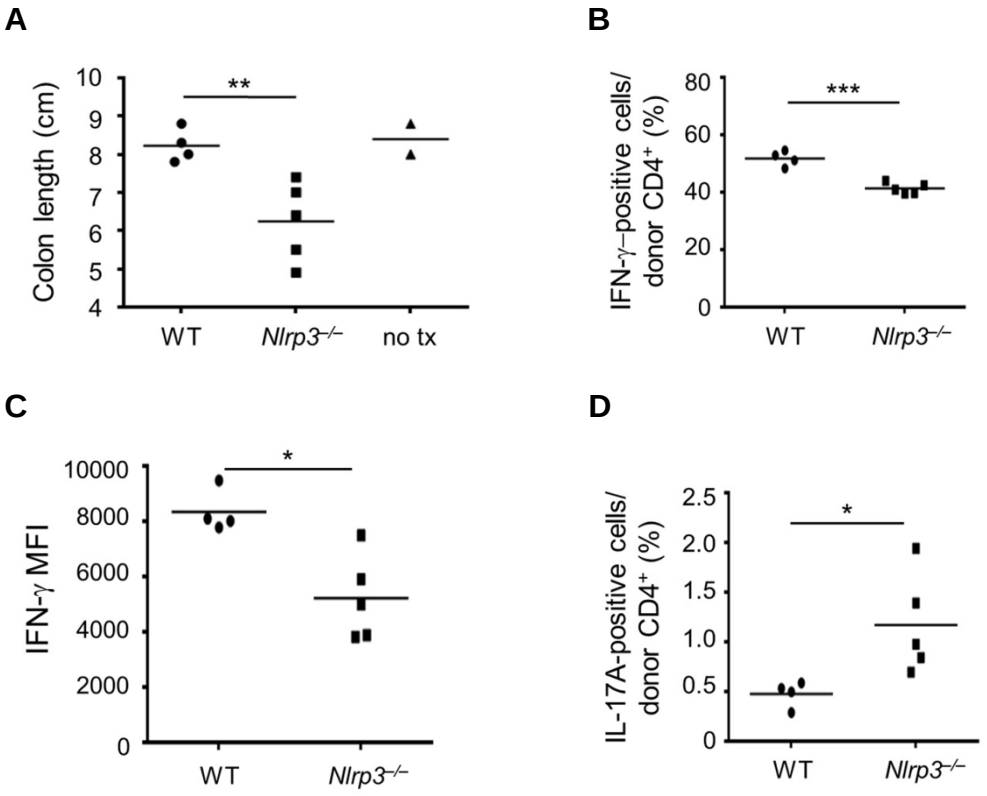

A
Colon length (cm)
10
9
8
7
6
5
4
**
WT
Nlrp3−/−
no tx
B
IFN-γ–positive cells/ donor CD4+ (%)
80
60
40
20
0
***
WT
Nlrp3−/−
C
IFN-γ MFI
10000
8000
6000
4000
2000
0
*
WT
Nlrp3−/−
D
IL-17A-positive cells/ donor CD4+ (%)
2.5
2.0
1.5
1.0
0.5
0
*
WT
Nlrp3−/−

Fig. S8

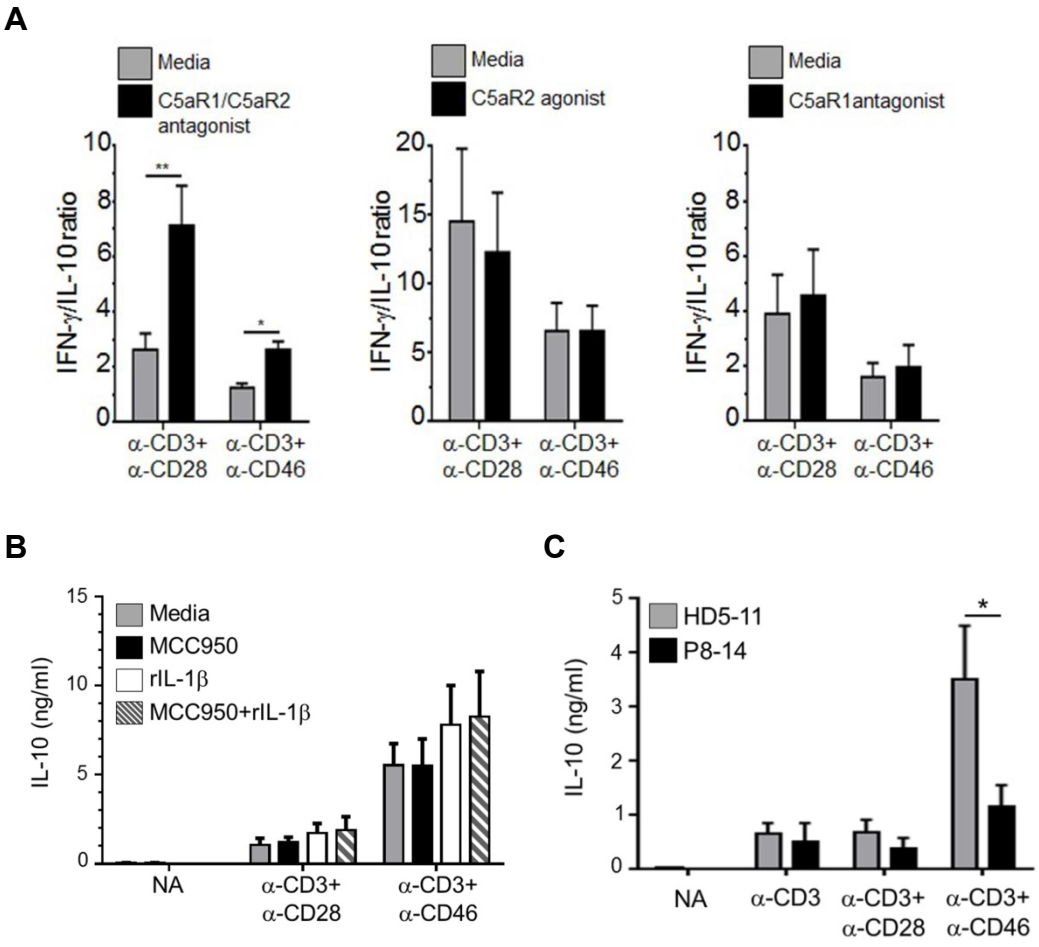
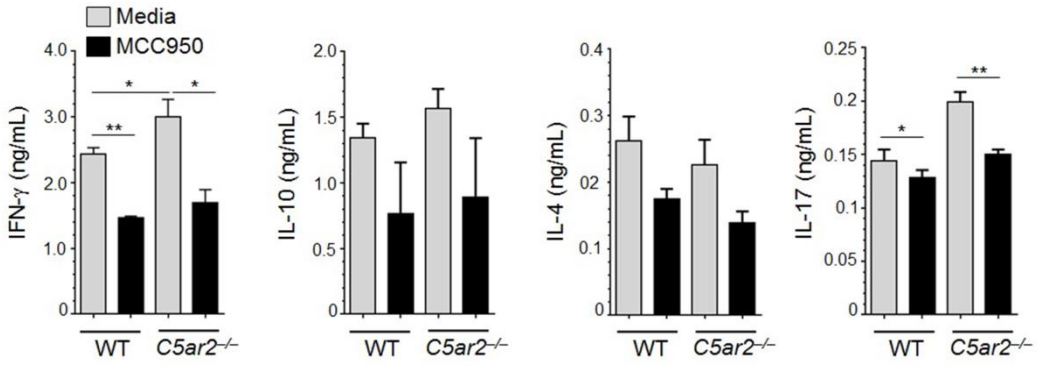

A
Media
C5aR1/C5aR2 antagonist
IFN-γ/IL-10 ratio
α-CD3+ α-CD28
α-CD3+ α-CD46
Media
C5aR2 agonist
Media
C5aR1antagonist
B
Media
MCC950
rIL-1β
MCC950+rIL-1β
IL-10 (ng/ml)
NA
C
HD5-11
P8-14
α-CD3

Fig. S9



2.5 Individual authors contributions (Arbore *et al.*, Science, 2016)

I have performed the large majority of the experiments using cells from healthy donors and patients and several mouse strains (see below), while Dr. Erin West from The National Heart, Lung and Blood Institute at the National Institutes of Health in Bethesda, MD (NHLBI, NIH) has performed all key mouse *in vivo* work and we thus shared the first authorship. Professor Claudia Kemper co-ordinated the whole study, gave substantial advice in experimental design and data interpretation and helped in writing and revising the manuscript. Below, the contributions of all co-authors are listed for any single main and supplementary figure of the manuscript.

Figure 1:

A to F - Giuseppina Arbore

G - Andreas Klos, Pavel Dutow, Claudia Rheinheimer

H - Giuseppina Arbore and Claudia Kemper

I - Giuseppina Arbore

(Additional input in form of reagents, experimental design and/or data interpretation: Jörg Köhl, Trent Woodruff and Pete Monk)

Figure 2:

A - Behdad Afzali

B - Behdad Afzali, Matthew Arno

C to H - Giuseppina Arbore

(Additional input in form of reagents, experimental design and/or data interpretation: Matthew Cooper, Luke O'Neill, Rebecca Coll, Avril Robertson)

Figure 3:

A - Giuseppina Arbore and Claudia Kemper

B to F - Giuseppina Arbore

(Additional input in form of experimental design and data interpretation: Helen Lachmann, Andrew Cope)

Figure 4:

A to G - Giuseppina Arbore

Figure 5:

A - Giuseppina Arbore

B - Claudia Kemper

C - Giuseppina Arbore

Figure 6:

A - Giuseppina Arbore

B and C - Erin West, Claudia Kemper, Katrin Mayer-Barber

D - Giuseppina Arbore and Erin West

E to H - Erin West and Katrin Mayer-Barber

(Additional input in form of reagents, experimental design and/or data interpretation: Warren Leonard, Alan Sher, Luke O'Neill, Matthew Cooper)

Figure 7:

A and B - Erin West and Rosanne Spolski

C - Zu Xi Yu

D and E - Erin West and Rosanne Spolski

Supplementary Figure 1:

A - Giuseppina Arbore

B - Giuseppina Arbore, Andreas Klos, Pavel Dutow, Claudia Rheinheimer

C - Giuseppina Arbore

D - Andreas Klos, Pavel Dutow, Claudia Rheinheimer

E - Claudia Kemper

F - Giuseppina Arbore and Claudia Kemper

G and H - Giuseppina Arbore

Supplementary Figure 2:

A - Behdad Afzali

B to J - Giuseppina Arbore

Supplementary Figure 3:

A - Giuseppina Arbore and Claudia Kemper

B - Giuseppina Arbore

Supplementary Figure 4:

A to E – Giuseppina Arbore

Supplementary Figure 5:

Claudia Kemper

Supplementary Figure 6:

A - Giuseppina Arbore

B - Erin West and Katrin Mayer-Barber

C and D - Erin West and Claudia Kemper

E and F - Erin West

G - Erin West and Claudia Kemper

H - Erin West

Supplementary Figure 7:

A to C - Erin West and Rosanne Spolski

D - Erin West

Supplementary Figure 8:

A to C - Giuseppina Arbore

Supplementary Figure 9:

Giuseppina Arbore, Claudia Kemper, Jörg Köhl

2.6 Discussion

The novel findings that came out of this thesis work are that: 1) C5 activation can occur intracellularly: 2) a canonical NLRP3 inflammasome is operative in $CD4^+$ T cells and required for normal Th1 responses; 3) the regulated crosstalk between intracellular C5 activity and the NLRP3 inflammasome is needed for the induction but also regulation of IFN-γ secretion by $CD4^+$ T cells.

Because a 'C3 system' in $CD4^+$ T cells had been described as key regulator of T cell effector functions previously (Cardone *et al.*, 2010; Liszewski *et al.*, 2013), the existence of an intracellular C5 activation system was likely. However, the expression of the C5aRs on mouse and human T lymphocytes is a matter of controversy in the field: work with green fluorescent protein (GFP) reporter mice suggested that the C5aR1 is not expressed in murine $CD4^+$ and $CD8^+$ T cells (Dunkelberger *et al.*, 2012; Karsten *et al.*, 2015). However, the insertion of fluorescent reporter proteins can transcriptionally destabilise the mRNA of the targeted gene (Reinhardt *et al.*, 2015) and, moreover several published papers endorse C5aR1 expression in mouse and human T lymphocytes (Nataf *et al.*, 1999; Strainic *et al.*, 2008; Lalli *et al.*, 2008; Strainic *et al.*, 2013; Kwan *et al.*, 2013). Thus, for the purposes of this thesis, particular emphasis was put on thoroughly and conclusively assessing the expression/presence of C5, C5a and the C5aRs on human T cells together with the help of leading experts and a solid range of technical approaches. C5 expression and low-level C5a generation were indeed observed in the resting state of T cells, while C5a intracellular generation increases upon *in vitro* activation (Figure 1 and supplementary Figure 1, Arbore *et al.*, 2016). Importantly, the mechanism mediating intracellular C5 cleavage in activated T lymphocytes has not been pursued in this study and warrants further investigations. As the Kemper laboratory has previously ruled out that cathepsin L (which activates C3 intracellularly) cleaves C5 (Liszewski *et al.*, 2013), candidates could include other proteases or possibly even an intracellular C5 convertase. The latter may be a real possibility as ongoing work in the laboratory by other members has indeed detected the generation of the Factor B neo-epitope (Bb), which is indicative of the alternative

pathway C3 convertase formation within T cells. Further, the data published demonstrate that at least in human $CD4^+$ T cells, the C5aR1 is expressed only intracellularly while the C5aR2 is found within cells and on the cell surface. Thus, this work provides the first conclusive description of intracellular C5 expression and activation as well as the C5a receptors expression pattern in human $CD4^+$ T cells – findings supporting a paradigm shift centralised around the notion that complement activation is not only confined to the extracellular space but occurs intracellularly and that $CD4^+$ T lymphocytes contain indeed a full intracellular complement system, the 'complosome' (Kolev *et al.*, 2014).

There is a long list of yet unanswered questions. Some key questions surrounding the C5aRs system that need to be addresses are: 1) the variations in protein expression and cellular localization of C5aR1 and C5aR2 (to assess by flow cytometry and confocal microscopy); 2) a possible C5aR1/C5aR2 interaction (to investigate by super-resolution microscopy and immunoprecipitation/immunoblotting techniques); 3) how the surface expressed C5aR2 negatively regulates Th1 immunity (see below); 4) the potential functions of the C5aR2 localised into the cytoplasm.

The subsequent functional analysis of the role of the intracellular C5aR1 and extracellular C5aR2 then demonstrated that the engagement of intracellular C5aR1 by C5a is required for induction of normal levels of IFN-γ (but not IL-4) and that the cell surface expressed C5aR2 (engaged by secreted C5a/C5adesArg in an autocrine fashion) negatively regulates IFN-γ production in human $CD4^+$ T cells (Figure 1 and supplementary Figure 1, Arbore *et al*., 2016). Interestingly, the fact that modulation of the T cell 'C5 system' did not affect IL-4 secretion (Th2 responses) aligns well with our current understanding that autocrine C3 activity is particularly important in the regulation of IFN-γ secretion (Liszewski *et al.*, 2013; Kolev *et al.*, 2014) – and indicates that both the autocrine C3 and the C5 system are needed for optimal Th1 immunity.

Gene array analyses using α-CD3 + α-CD46 versus α-CD3 + α-CD46 + dRA activated human $CD4^+$ T cells, which were supposed to give a hint about the pathways regulated by the C5 system that is driving IFN-γ, revealed *NLRP3* and *IL1B* gene expression by activated human $CD4^+$ T lymphocytes (Figure 2, Arbore *et*

al., 2016). This observation was unexpected because so far canonical NLRP3 inflammasome activity has not been described in adaptive immune cells. Myeloid and particularly monocytes and macrophages were thought to be major NLRP3 inflammasome-operative cells as they are also the largest producers of IL-1β (Dinarello, 1996, Rao *et al.*, 2007). Although there was indication that NLRP3 expression may be more ubiquitous as it was also later found in endothelial cells, epithelial cells and neurons (Heneka *et al.*, 2013; Shahzad *et al.*, 2015; Cao *et al.*, 2016), early reports on potential presence of NLRP3 in T cells were dismissed and thought to be contaminations with monocytes.

However, *in vitro* and *ex vivo* work carefully performed with T cells from healthy donors and from CAPS patients showed then clearly the 'classical' function of an NLRP3 inflammasome in human $CD4^+$ T cells with the secretion of mature IL-1β as 'end product' (Figure 2 to 4 and supplementary Figure 2 to 4, Arbore *et al*., 2016). Further, full activation of the NLRP3 inflammasome requires C5aR1-driven ROS activation (Figure 5 and supplementary Figure 5, Arbore *et al*., 2016). These observations align with the previous knowledge that the C5aR1 is a key ROS inducer in monocytes and neutrophils (Guo *et al.*, 2003; Daniel *et al.*, 2006). It was, however, always assumed that cell surface C5aR1 activation drives ROS production in these cells – and the data observed during this thesis work suggest that it may be worthy to assess if ROS production in monocytes and neutrophils may also be (at least in part) dependent on intracellular C5a activity. This may be important for sterile inflammation as C5aR1 triggers augmented ROS generation upon cholesterol crystal uptake and induced the inflammasome in myeloid cells (Samstad *et al.*, 2014) - and there is now indeed indication that the intracellular C5aR1 is instrumental in these cells (Kemper laboratory, unpublished data).

Nonetheless, is also likely that other complement-mediated pathways (aside from ROS) contribute to the modulation of the NLRP3 inflammasome function in T cells. A candidate initially considered as potential C5a-driven inflammasome activator in T cells was the S100A8/S100A9 (calprotectin), a TLR4 ligand (Ehrchen *et al.*, 2009), because S100A8/S100A9 expression was also modulated in T cells from individuals with complement dysregulation (data not shown). This was indeed an attractive

candidate initially because a mechanistic interplay between complement and S100A8 has previously been reported, where C5a can stimulate the release of S100A8 from PBMCs (Hetland *et al.*, 1998), while with regards to inflammasome activity S100A8 is also a molecular marker of CAPS disease progression (Wittkowski *et al.*, 2011) and a driver NF-kB activation and ROS production, thus activating the NLRP3 inflammasome (Simard *et al.*, 2013). Following these considerations, immunoblot and immunostaining analysis on human $CD4^+$ T cells proved indeed S100A8 protein expression, which is higher in the activated status and increased by C5aR2 antagonism (dRA) but not by MCC950 addition (data not shown), in support of the hypothesis that this ligand is regulated by autocrine C5a-mediated pathways upstream of inflammasome activation. However, additional experiments performed in the course of this thesis project were unable to reduce S100A8/S100A9 protein expression by silencing RNA technique and could, thus, not assess its function within the C5/NLRP3 inflammasome axis in T cells.

A second protein of interest is the inflammasome assembly regulator GBP5 (Shenoy *et al.*, 2012), as *GBP5* is among the most up-regulated genes by $CD4^+$ T cells activated upon C5aR2 antagonism (data not shown). Although GBP5 expression has not yet been shown in T cells, it has recently been identified as a 'rheostat' of NLRP3 inflammasome activation in mouse monocytes (Shenoy *et al.*, 2012). Thus, the functional connection between C5a and GBP5 expression may be fruitful area of research for the future.

Of course, the definition of how C5aR2 may negatively regulate these events is of critical importance as it would possibly deliver the means for new therapeutic interventions for T cell-mediated diseases: extracellular engagement of C5a/C5adesArg to C5aR2 down-modulates the NLRP3 inflammasome either directly via a yet unidentified mechanism or indirectly by inhibiting intracellular C5aR1. C5aR2 is likely to achieves this inhibitory effect via engagement of β-arrestins: noteworthy, ARRB2 can interact with other GPCRs, such as GPR40 and GPR120 activated by omega-3 fatty acids (Yan *et al.*, 2013) and the lactate receptor GPR81 (Hoque *et al.*, 2014) to dampen NLRP3 inflammasome activation; in addition, ARRB2 can associate with C5aR2 to inhibit C5aR1-driven ERK1/2 signaling (Croker *et al.*, 2014). Thus, it would be interesting to assess in the future if these pathways are engaged in T cells upon C5aR2 stimulation and regulate Th1 activity.

Regarding a role for autocrine C5 system in mouse, higher in vitro IFN-γ secretion has been observed in C5aR2 deficient $CD4^+$ cells, which is normalised by the inflammasome inhibitor MCC950, demonstrating an anti-inflammatory role for this receptor (supplementary Figure 9, Arbore *et al*., 2016). Conversely, the notion that autocrine C5a-driven C5aR1 positively regulates Th1 responses is supported by other studies showing a clear defect in IFN-γ production by $CD4^+$ T lymphocytes from *C5*$^{-/-}$ (Mashruwala *et al.*, 2011) or *C5ar1*$^{-/-}$ mice (Strainic *et al.*, 2008). However, there are differences among the mouse and human species in expression of complement receptors and regulators (see Chapter 1) and this is most clearly demonstrated for CD46 (Kolev *et al.*, 2014), therefore making difficult to compare the complement contribution to inflammasome activity in T cells among the mouse and human species.

Another surprising observation coming out of this work is that autocrine IL-1β production by $CD4^+$ T cells is critical for normal *in vivo* Th1 induction (Figure 6 and 7 and supplementary Figure 6 and 7, Arbore *et al*., 2016). $CD4^+$ T lymphocytes from WT mice treated with MCC950 or isolated from mice deficient for inflammasome components (*Nlrp3*$^{-/-}$, *Il1a/b*$^{-/-}$ or *Il1r1*$^{-/-}$) showed reduced IFN-γ production upon *in vitro* activation, without an effect on IL-4, IL-17 and IL-10 cytokines and on cell viability. But importantly, in all three *in vivo* experiments (the LCMV infection, the colitis and the GvHD models), *Nlrp3* KO T cells (or T cells deficient in *Il1b or Il1r1*) (Mayer-Barber *et al.*, 2014) were injected into animals in which all other cells were fully capable of making IL-1β in normal amounts. Nonetheless, IFN-γ production was decreased by about half in all models - and neither the expression of the key Th1 lineage transcription factor T-bet (Lugo-Villarino *et al.*, 2003) nor cell viability were affected (unpublished data and Arbore *et al.*, Science, 2016).
Although in the experiments for this thesis it has been observed basically no direct intrinsic effect of the canonical NLRP3 inflammasome on Th2 and Th17 cytokines production *in vitro* and *in vivo* (supplementary Figure 2 and 6, Arbore *et al*., 2016), it should be noted that exogenous IL-1β is known to boost also Th2 and Th17 responses apart from Th1 (Ben-Sasson *et al.*, 2009). Thus, IL-1β effects in general on T cell responses may be context dependent. In fact, intrinsic NLRP3 protein (but not inflammasome) activity in $CD4^+$ T cells has been shown to act as transcriptional

activator of Th2 differentiation (Bruchard *et al.*, 2015) and to drive Th17 response in vivo in EAE as part of an inflammasome complex (Martin *et al.*, 2016). Noteworthy, both these studies investigated Th1, Th2 and Th17 *in vitro* responses under exogenous skewing stimuli, while in the work here reported, for both human and mouse *in vitro* data, the experimental set-up throughout is not traditional in vitro Th1 skewing with IL-12 and anti-IL-4 (to avoid 'masking' any observations by addition of non-physiological levels of cytokines) but activation of $CD4^+$ T cells with anti-CD3 and co-stimulation under non-polarising conditions. Furthermore, in many published studies using mouse systems, NLRP3 inflammasome-driven IL-18 production also contributes heavily to Th1 responses while our data indicate that at least human $CD4^+$ T cells do not produce IL-18 and do not rely on an intrinsic function of this cytokine (supplementary Figure 2J, Arbore *et al*., 2016).

Given that APCs provide generally ample amounts of IL-1β during the cognate APC/T cell interaction, the finding that normal IFN-γ production requires also T cell autocrine IL-1β production was unexpected. The exact reasons for this are not yet clear but it is likely that, while APC-derived NLRP3-activated IL-1β supports initial Th1 priming, successful 'imprinting' or maintenance of the Th1 phenotype during differentiation and migration into the periphery may require autocrine NLRP3 activity. It would therefore be a valuable experiment to assess if autocrine IL-1β may impact on epigenetic changes in the DNA landscape.

IL-1β production by T cells, in comparison to myeloid cells, is relatively low and tightly regulated by an autocrine C5aR1 versus C5aR2 activation balance. The probable reason for this is that rapid control of local IL-1β is critical to normal termination of Th1 responses: human Th1 cells co-induce IL-10 secretion in a CD46-dependent fashion (section 1.2.4 of this thesis) during their contraction phase and failure of this 'IL-10 switch' underlies hyperactive Th1 responses observed in rheumatoid arthritis and multiple sclerosis (Astier *et al.*, 2006; Cardone *et al.*, 2010). IL-1β strongly suppresses IL-10 production by effector Th17 cells (Zielinski *et al.*, 2012) and, accordingly, it has been observed that blockade of C5aR2 increases the IFN-γ:IL-10 ratio in $CD4^+$ T cells while IL-1β addition to the activated cells increases IFN-γ but blocked proportional IL-10 secretion. Fully in line with this, T

cells from CAPS patients have significantly reduced IFN-γ to IL-10 switching (supplementary Figure 8C, Arbore *et al*., 2016).

The adoptive T cell transfer models using *Nlrp3* deficient $CD4^+$ T cells, to induce colitis and acute GvHD disease in mice (Figure 7 and supplementary Figure 7, Arbore *et al*., 2016), gave also a very surprising finding. It was initially expected that mice injected with the *Nlrp3* deficient $CD4^+$ T cells would have less severe disease or later onset because of reduced Th1 responses, which are acknowledged to play a role in these settings (Powrie *et al.*, 1994; Nikolic *et al.*, 2000). However, the mice injected with the *Nlrp3* deficient $CD4^+$ T cells had clearly more severe disease in both models when compared to animals injected with WT cells. Assessment of other cytokines affected in these models then showed that the reduced *in vivo* Th1 induction (due to lack of intrinsic NLRP3 inflammasome activity in $CD4^+$ T lymphocytes) tips the balance towards Th17 responses and via this causes more severe disease. In full concordance with the finding that Th1 and Th17 responses counter-regulate each other, activated $CD4^+$ T cells from CAPS patients (overactive NLRP3) produce higher IFN-γ and lower IL-17 *in vitro* (Figure 3E, Arbore *et al*., 2016). This may also explain why many patients fail to have obvious T cell-mediated autoimmune symptoms. My findings also explain why the role of NLRP3 was discussed so controversial in the field of IBD: Th17 response is associated with colitis (Coccia *et al.*, 2012) and regulated by paracrine IL-1β from APCs and in one study, lack of Nlrp3 indeed reduced disease severity (Bauer *et al.*, 2010). However, similarly to the data herein shown, Hirota and colleagues (2011) observed higher susceptibility to colitis in *Nlrp3* KO mice (however colitis was induced by DSS exposure and not $CD4^+$ cell transfer, not discriminating the role for NLRP3 in different myeloid and lymphoid cell subsets), although discordant studies have been reported (Bauer *et al.*, 2010). Thus, it is now clear that it is important in which cell type NLRP3 activity is modulated and there are obvious different effects of paracrine and autocrine IL-1β functions in the intestine. These considerations led to the start of a follow-up project in collaboration with the clinicians Dr. Nick Powell and Polychronis Pavlidis (from King's College London) to investigate whether the intrinsic $CD4^+$ T cells complement/inflammasome axis partakes also in colitis

pathogenesis in humans, specifically in patients with Crohn's disease, with the possibility to modulate this system therapeutically – this work is currently ongoing.
Of note, additional attempts were made to address an *in vivo* role for hyper-active NLRP3 inflammasome activity of $CD4^+$ T cells in humans, performing a GvHD experiment with the adoptive transfer of naïve $CD4^+$ cells isolated from patients with CAPS into *Rag2*$^{-/-}$ mice in collaboration with Dr Nick Powell and Dr Adam Laing (King's College London). Although engraftment of human $CD4^+$ T cells was effective with detection in mouse serum of human IFN-γ, with a trend toward higher cytokine over percentage of engraftment in recipients of patients T cells when compared to recipients of HDs cells, mice did not show weight loss after two months from T cell transfer (data not shown). Given the fact that T cells from CAPS patients produce less IL-17 (Figure 3E, Arbore *et al*., 2016) and that IL-17 production is critical in GvHD, the mice may have not developed disease because of reduced production of this cytokine by human $CD4^+$ T cells.

During my MSc studies in the Kemper laboratory, I participated in a project that ultimately led to the finding that autocrine CD46 activation during T cell stimulation is required for metabolic reprogramming during Th1 cell responses (Kolev *et al.*, 2015). Upon TCR activation, CD46 is engaged via T cell autocrine C3b production and induces (aside from *IL1B* and *NLRP3* gene expression) also the increased expression of the glucose and AA channels, GLUT1 and LAT1, respectively, thereby mediating the nutrient influx needed for T cell activation (Kolev et al., 2015) Moreover, CD46 also up-regulates the late endosomal/lysosomal adaptor, MAPK and MTOR activator 5 (LAMTOR5), which then drives mTORC1 assembly and activation with subsequent increased glycolysis (Kolev *et al.*, 2015), in line with previous work that has linked augmented glycolysis in human $CD4^+$ T cells with IFN-γ production (Marelli-Berg *et al.*, 2012). Thus, this work demonstrates that the autocrine C3 system in T cells participates in key cell metabolic pathways.
Now, the published work on the functional interplay between the 'cellular complosome and the inflammasome' (Arbore *et al*., Science, 2016) brings together the 'intracellular C5/C5a/C5aR/NLRP3-inflammasome axis' and the already described 'CD46/C3/C3a/C3aR-metabolism axis' in T cells (Kolev *et al.*, 2013). CD46 participates in NLRP3 inflammasome priming: CD3 and CD46 co-stimulation

leads to upregulation of inflammasome genes, and in addition previous work in Kemper lab demonstrated that CD46 engagement potentiates NF-kB activation in stimulated human $CD4^+$ T cells (Kolev *et al.*, 2015). Moreover, CD46 activation drives mTORC1 activity, nutrient influx and augmented glycolysis (Kolev *et al.*, 2015), a metabolic change fundamental for T cell effector functions (Marelli-Berg *et al.*, 2012), while mTORC1 activity and glycolysis have been recently shown as NLRP3 inflammasome inducers in macrophages (Moon *et al.*, 2015). Accordingly, treatment of T cells with the mTORC1 inhibitor rapamycin (Kolev *et al.*, 2015) during CD46-activation abolishes caspase-1 activation, IL-1β and IFN-γ secretion (data not shown), therefore providing other signals for inflammasome induction (Figure 2.2). Thus, the cross-talk between intracellularly activated complement components, the 'complosome' (Kolev *et al.*, 2014), key metabolic changes and the NLRP3 inflammasome emerge as fundamental to human Th1 induction and modulation. Detailed background information and an in-depth discussion on the potential significance for health and disease of this novel 'Complement-metabolism-inflammasome axis' is included in a recent Mini-Review in the *European Journal of Immunology* of which I am the 1st author (Arbore and Kemper, 2016).

In summary, two quintessential innate pathways, the intracellular complosome and the inflammasome, are fundamental contributors to the adaptive immune system. That established innate immune pathways previously unpostulated to be operative in adaptive immune cells are not only present but also key in directing immunological responses is of great significance to our understanding of the immunobiology and why targeting just one arm of the immune system is often insufficient for the treatment of human diseases. This complement/metabolism/inflammasome axis may constitute a bull's eye therapeutic approach for the treatment of autoimmune and autoinflammatory conditions, metabolic disorders and cancer – as it is possible that this axis is also operative in a wide range of other immune cells.

Finally, it is worth to explore whether autocrine complement-NLRP3 inflammasome activity is also required for optimal IFN-γ production by other immune cell subsets, such as $CD8^+$ T lymphocytes, natural killer T (NKT) cells and innate lymphoid type 1 (ILC1) cells. Within the PhD project, after work on $CD4^+$ T cells, preliminary data

were generated on human $CD8^+$ cytotoxic T cells with regards to C5 system and NLRP3 on IFN-γ production and effector functions, which constitute the Chapter 3 of this thesis.

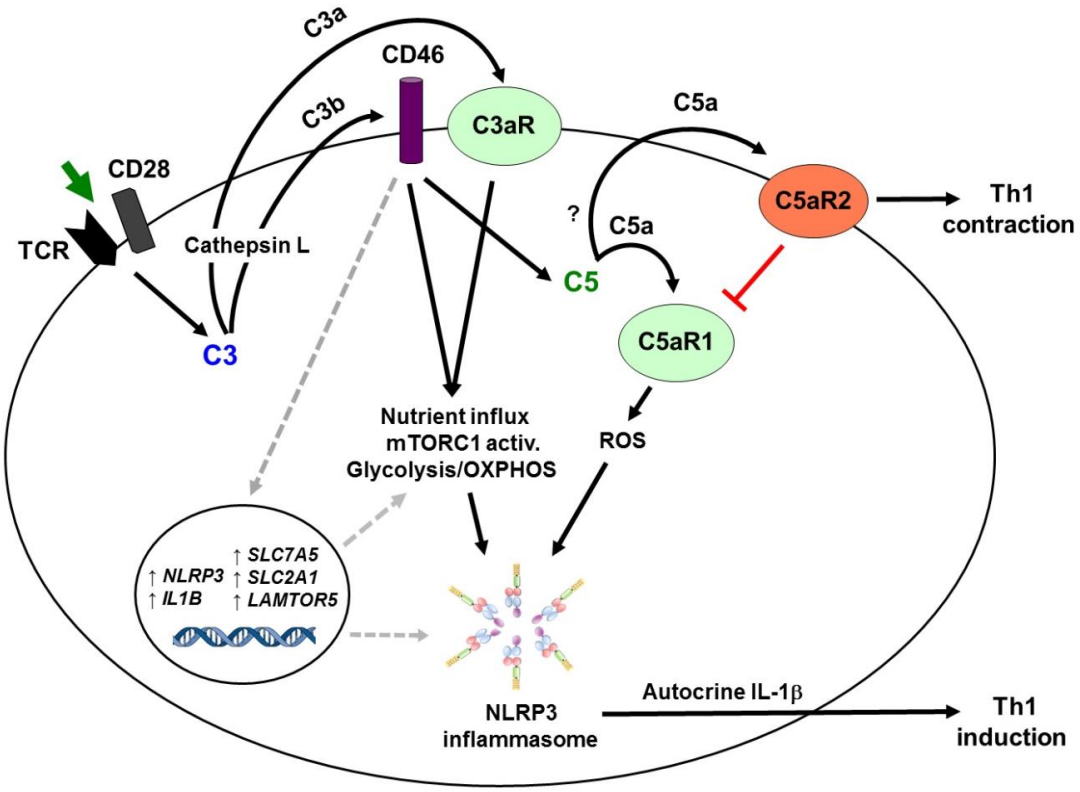


Figure 2.2 A complement-metabolism-inflammasome axis regulates human Th1 response induction and contraction
TCR stimulation of human $CD4^+$ T cells induces the autocrine activation of CD46 and the C3aR via the 'C3 system' that cumulates in nutrient influx, mTORC1 activation (which is a NLRP3 inflammasome activator) and induction of key metabolic events. In addition, CD46 stimulation simultaneously induces gene expression of NLRP3 and IL1B to prime the NLRP3 inflammasome as well as increased intracellular C5 activation and C5a generation (of note, the enzyme cleaving intracellular C5 into C5a and C5b has not yet been identified and is denoted by a question mark). Intracellularly generated C5a then engages the intracellular C5aR1 to amplify ROS production. Increased ROS levels, together with the indicated metabolic changes, induce the assembly of the NLRP3 inflammasome and subsequent IL-1β (but not IL-18) production required for optimal Th1 induction. Cell surface expressed C5aR2 (engaged via secreted C5a/C5adesArg) negatively regulates C5aR1 signaling via a yet-unidentified mechanism - and therefore controls the Th1 responses.
From Arbore and Kemper, 2016

Chapter 3

Results and Discussion Part 2: Autocrine complement and NLRP3 inflammasome in human $CD8^+$ T cells

3.1 Introduction

3.1.1 Complement-mediated regulation of $CD8^+$ T cells responses

Although the larger body of work about the complement's role in T cell immunity has been performed on $CD4^+$ T cells, there are also a number of studies showing that local complement modulates the cytotoxic activity of $CD8^+$ T lymphocytes *in vitro* and *in vivo* in response to infection and during anti-cancer responses.

Their ability to respond to complement products is demonstrated by the fact that $CD8^+$ T cells express the complement receptors and regulators, such as CD46, DAF and CD59 whilst the expression of CR1 has not been reported (Christmas *et al*. 2006). Further, *in vitro* stimulation of human $CD8^+$ T lymphocytes with phytohaemagglutinin leads to upregulation of surface-expressed CD46 and DAF, thus suggesting that these two molecules may also play a role in shaping $CD8^+$ T lymphocytes immune responses.

The first studies which showed a role for CD46 costimulation in promoting $CD8^+$ T cell proliferation, and in induction of morphological changes associated with activation, analysed total $CD3^+$ T lymphocytes without initial subsorting into $CD4^+$ and $CD8^+$ subpopulations (Astier *et al*., 2000, Zaffran *et al*., 2001), therefore indicating that CD46 may also promote $CD8^+$ T cells effector functions aside from $CD4^+$ T cells.

Several experimental observations demonstrate that indeed CD46 co-activation modulates T cell mediated cytotoxicity. Firstly, in a mouse model of contact hypersensitivity reaction in mice either expressing human CD46 CYT-1 or CYT-2 tail bearing isoforms, CD46 engagement controls total splenocytes cytotoxicity, with CYT-1 bearing isoform having a negative and CYT-2 a positive effect (Marie *et al*, 2002). This observation is in opposition with human data on $CD4^+$ T cells where CYT-1 promotes pro-inflammatory response and CYT-2 activity allows Th1 contraction (Cardone *et al*., 2010, Kolev *et al*., 2015), however this discrepancy might be explained by the fact that mouse T cells lack the exactly similar signaling

machinery required for normal human CD46 function and hence distinct signaling events are induced by hCD46 in mouse T cells and in human T cells (Yamamoto *et al*., 2013). Secondly, CD46 co-stimulation on human $CD4^+$ T cells leads surprisingly to granzyme B and perforin overexpression, an event which allows the contact-dependent cytotoxic elimination of autologous activated T lymphocytes by $CD4^+$ T cells (Grossman *et al*., 2004). Thirdly, CD46 ligation by anti-CD46 coated beads leads to microtubule polarization and reduced IFN-γ production in $CD8^+$ cytotoxic T lymphocytes, in addition to altered perforin polarization and cytotoxicity to the target cell (Oliaro *et al*., 2006), although in contrast to the positive effect ascribed for CD3 and CD46 co-ligation on a solid surface in T cells (Astier *et al*., 2000, Kemper *et al*., 2003).

Apart from a general effect on T cell cytotoxicity, most recently CD3 and CD46 costimulation of $CD8^+$ T cells has been demonstrated to increase cell proliferation and induce the expression of several T cell activation markers, such as CD25, CD28, OX40 and PD-1 (Kickler *et al*., 2012). Importantly, this latter study did not demonstrate a significant effect of CD46 co-stimulation on IFN-γ production by $CD8^+$ T cells, albeit a thorough analysis of the effects of CD46 activation on different T cell subsets and level of TCR stimulation as controls had not been performed in that study. Thus, further studies are needed to better elucidate the potential role of CD46 on human $CD8^+$ T lymphocytes function.

In addition to CD46, signaling mediated by the anaphylatoxins C3a and C5a appears to shape $CD8^+$ T cell migration and effector function.

Expression of the C3aR has been detected not only in human $CD4^+$, but also in $CD8^+$ T cells (Werfel *et al*., 2000). Further studies also demonstrated the ability of cytotoxic T cells to respond to complement C3 activation: local C3 generation and activation was required for T cell-mediated control of influenza virus infection in a mouse model, where *C3* gene KO mice showed reduced cytotoxic T lymphocytes priming in the draining lymph nodes and impaired recruitment in the lung of virus-specific IFN-γ producing T cells, independently from CR2 expression (Kopf *et al*., 2002). A similar C3-dependent, CR2-independent, mechanism in anti-viral immune response has also been observed in a model of acute LCMV infection, where it has been demonstrated that complement C3 activity regulates epitope selection and is

needed for optimal expansion of LCMV-specific mouse effector $CD8^+$ T lymphocytes (Suresh *et al*, 2003).

The current opinion is that complement-mediated regulation of $CD8^+$ T cells effector functions is rather operating via C5 complement activation products, as C3 activation generates the C3b component of C5 convertase (which cleaves C5 into C5a and C5b) and *Cr2*$^{-/-}$ mice did not show defective anti-viral $CD8^+$ T cell response (Suresh *et al*, 2003). Such notion is in line with other studies indicating the importance of local C5a in shaping $CD8^+$ T cell immunity *in vivo*. In a model of flu infection, mice treated systemically with the C5aR1 antagonist PMX53, which blocks C5a binding to this receptor, showed diminished frequency of flu-specific $CD8^+$ T cells (Kim *et al.*, 2004b). Further, CD55 (DAF) expression increases in human $CD8^+$ lymphocytes upon *in vitro* stimulation (Christmas *et al.*, 2006), and mice deficient for *Daf1*, which leads to reduced formation of complement convertases on the T cell surface, demonstrated heightened $CD8^+$ T cell immunity to LCMV infection, which is reversed by co-deletion of either C3 or C5aR1 (Fang *et al.*, 2007). Several mechanisms by which C5a can modulate the priming of $CD8^+$ T cells have been proposed, such as indirect T cell activation by paracrine downstream inflammation products, via increased recruitment and stimulation of antigen presenting cells, or direct enhancement of T cell trafficking or $CD8^+$ TCR antigen sensitivity (Kim *et al*. 2004b). The effects of locally produced C5a on $CD8^+$ T lymphocytes also affects the outcome of T cell-mediated graft injury *in vivo*: *Daf1*$^{-/-}$ mice which receive a heart transplant undergo increased $CD8^+$ T cell-dependent $CD4^+$ T cell-independent graft rejection compared to WT recipients. This enhanced $CD8^+$ T cell reactivity may be due to the augmented stimulation by APCs, as locally produced C3a and C5a up-regulate the expression of CD80 and CD86 by APC (thus facilitating antigen presentation) and increase IL-12 production (Vieyra *et al.*, 2011). Further studies in mice showed that paracrine-derived C5a from endothelial cells stimulates cytotoxic T cell expansion, IFN-γ and perforin production (Raedler *et al*., 2009), while $CD8^+$ T cells lacking C3aR and C5aR1 proliferate weakly to allogeneic dendritic cells (which produce C3a and C5a) (Vieyra *et al*., 2011). Of note, these latter two studies suggest an effect of C3a and the C3aR and indicate that C3 activation products (and not just C5 activation products) also contribute to $CD8^+$ T cell immunity.

Interestingly (and somewhat similar to the $CD4^+$ T cell situation), local complement can also have negative impact on $CD8^+$ T cell responses: C5a appears to indirectly inhibit $CD8^+$ T cell anti-tumor responses. In a syngeneic mouse model of cervical cancer, blockade of C5aR1 via subcutaneously injected PMX53 results in reduced tumor growth, while locally produced C5a favours the recruitment of myeloid-derived suppressor cells (MDSC), which suppress T cell immunity (Markiewski *et al*., 2008). This C5a-mediated MDSC suppression of anti-tumor $CD8^+$ T cell responses is also associated with augmented formation of cancer metastases: in a mouse model of breast cancer, mice either deficient for C5aR1 or treated with the C5aR1 antagonist PMX53 show reduced metastasis formation and higher frequency of IFN-γ producing *ex vivo* restimulated $CD4^+$ and $CD8^+$ T cells isolated from the lung (Vadrevu *et al*., 2014). It seems that the concentration of C5a within the tumor microenvironment determines the anti-tumor T cell responses and cancer progression: tumor-bearing mice with high C5a-producing syngeneic lymphoma cells had significantly accelerated disease progression and decreased proportion of infiltrating $CD4^+$ and $CD8^+$ T lymphocytes in the tumor, tumor-draining lymph nodes, and spleen, while mice recipient of low C5a-producing lymphoma cells had significantly diminished tumor burden and increased numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes (Gunn *et al*, 2012). Finally, recent evidence suggests a role for local C3a and C5a in inhibiting intrinsic IL-10 production and anti-tumor activity by $CD8^+$ tumor infiltrating lymphocytes in a breast cancer mouse model, therefore anaphylatoxin receptor signaling can dampen anti-tumor T cell immunity, favouring disease progression (Wang *et al*., 2016).

The mechanism by which C5a anaphylatoxin regulates $CD8^+$ effector functions in vivo, with opposing and different effects in anti-viral and anti-tumor immunity, have not yet been fully elucidated. Particularly, if C5a acts directly through C5aR1 expressed by $CD8^+$ T cells or indirectly on APCs or myeloid cells expressed C5aR1 to shape T cell immunity is still a matter of controversy.

Expression of C5aR1 mRNA has been detected by total ($CD4^+$ and $CD8^+$) human peripheral blood T lymphocytes (Nataf *et al.*, 1999) and by mouse $CD8^+$ cytotoxic T cells (Raedler *et al*., 2009). C5aR1 protein expression has been detected only in effector (not resting) human $CD8^+$ T cells: while T cell stimulation with

phytohaemagglutin leads to the generation of a C5aR1$^+$CD3$^+$ cell population (Nataf *et al*., 1999), tumor infiltrating CD8$^+$ T lymphocytes from patients with melanoma, but not from the peripheral blood, show cell-surface expressed C5aR1 (Wang *et al*., 2016). This is in line with work from Vadrevu and collaborators, which did not detect any C5aR1 protein expression from the peripheral blood mouse CD8$^+$ T cells (Vadrevu *et al*., 2014).

Moreover, an effect for the alternative C5a receptor C5aR2 in shaping CD8$^+$ T cell immunity has not been investigated by any of these studies.

A better understanding of C5a mechanism of action, either paracrine or autocrine, on C5a receptors expressed by either APCs or CD8$^+$ lymphocytes, may elucidate the role of local complement in modulating the antigen-specific killing of infected or tumor cells.

Thus although the exact molecular pathways driving the effects of C5a are not defined yet, there is strong indication that cytotoxic anti-viral T cell responses can be modulated via targeting C5a mediated signaling in mice and humans.

3.1.2 Inflammasome-mediated regulation of CD8$^+$ T cells responses

Apart from signals derived from complement activation products, it is now acknowledged that signals coming from APCs and myeloid cells-derived pro-inflammatory IL-1β and IL-18 cytokines, produced as result of inflammasome activation, are critical regulators of CD8$^+$ T cells cytotoxicity (Kupz *et al*., 2012, Ben-Sasson *et al.*, 2013).

In recent years, IL-1β has been defined as a strong enhancer of antigen-specific CD8$^+$ T cell responses *in vivo*. IL-1β facilitates their expansion, tissue migration, effector functions and memory responses (Ben-Sasson, *et al*., 2013): 1) mice receiving ovalbumin-specific transgenic CD8$^+$ T cells and then immunised with ovalbumin and LPS, showed increased accumulation of antigen-specific CD8$^+$ T cells in the peripheral tissues (spleen, liver and lung) in the presence of exogenous IL-1β and this effect is not observed upon transfer of ovalbumin-specific CD8$^+$ T lymphocytes deficient for the IL-1R1, 2) IL-1β administration results in heightened IFN-γ and granzyme B production by antigen-specific CD8$^+$ T cells both upon

primary immunization and antigen re-challenge (memory response); 3) IL-1β co-administration favours *in vivo* T cell cytotoxicity to inoculated splenocytes loaded with ovalbumin-tetramers. It is likely that IL-1β/IL1R1 signaling enhances antigen-stimulated $CD8^+$ T cell expansion via suppression of apoptosis, a mechanism which seems operative in IL-1β-driven increase of $CD4^+$ T cell responses (Ben-Sasson *et al*., 2009).

At least in the models assessed, the IL-1β-mediated effect on *in vivo* $CD8^+$ T cell responses is thought to depend on paracrine activation of the NLRP3 inflammasome in APCs and subsequent production of IL-1β and this aligns with data reporting that the NLRP3 inflammasome activity and the IL-1β production promote anti-tumor T cell cytotoxicity in a mouse model of thymoma (Ghiringhelli *et al*., 2009). However, an NLRP3-independent and IL-1β-dependent $CD8^+$ protective T cell response has been demonstrated *in vivo* in flu models and an intrinsic activity for the NLRP3 inflammasome in $CD8^+$ T cells has so far not been assessed (Ichinohe *et al*., 2009).

The inflammasome-derived cytokine IL-18 can also enhance IFN-γ production by T lymphocytes (Okamura *et al*., 1995). Again, published work suggests that APCs-derived IL-18 is critical for $CD8^+$ T cell responses, and particularly effector memory function (Soudja *et al*., 2012, Kupz *et al*., 2012). Soudja and collaborators showed that the canonical NLRP3 inflammasome pathway contributes to *in vitro* and *in vivo* IFN-γ production by memory $CD8^+$ T cells: while exogenous IL-18 enhances ex-vivo IFN-γ response by $CD8^+$ T lymphocytes (possessing an ovalbumin-specific TCR and isolated from mice pre-challenged with ovalbumin) in absence of antigen stimuli, bone-marrow caspase-1 deficient mice (which lack full canonical inflammasome activity) showed a significantly diminished proportion of IFN-γ^+ $CD8^+$ T antigen-specific memory cells upon antigen immunization when compared with WT animals. The authors demonstrated further that a subset of inflammatory monocytes represents the source of IL-18 which initiates $CD8^+$ T cells differentiation and effector responses to bacterial, viral and parasitic infections (Soudja *et al*., 2012). NLRC4 inflammasome driven IL-18 production by DCs can also support antigen-independent IFN-γ production by memory $CD8^+$ T cells (Kupz *et al*., 2012).

Overall, the *in vitro* and *in vivo* studies published so far support a clear role for paracrine NLRP3 (and NLRC4) inflammasome activation in APCs that drives IL-1β

and IL-18 production which in turn supports $CD8^+$ T lymphocyte effector function and memory development. The possibility of an intrinsic functional NLRP3 inflammasome machinery (possibly regulated by intracellular complement) that is required for normal $CD8^+$ T cell function (as herein demonstrated for $CD4^+$ T cells) has so far not been evaluated.

3.2 Hypothesis and aims

As main part of this thesis project, data have been generated which demonstrated that complement C5-regulated canonical NLRP3 inflammasome activity within $CD4^+$ T lymphocytes is required for optimal IFN-γ production and Th1 responses *in vitro* and *in vivo* (Chapter 2). As $CD8^+$ T cells, like $CD4^+$ T cells, also produce IFN-γ and granzyme B and can respond to CD46 engagement and C5aR1 stimulation but the downstream events of the engagement of these regulators/receptors is not defined, the further hypothesis has been developed and investigated:

'Human $CD8^+$ T cells, similar to $CD4^+$ T cells, harbour an intracellular C3 and C5 system and this system contributes to effector responses in direct cross-talk with an intrinsic NLRP3 inflammasome'.

3.3 Materials and methods

3.3.1 Cells isolation, culture and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors (HDs) (in accordance with the guidelines of the Wandsworth Research Ethics Committee, REC number 09/H0803/154) by centrifugation with Ficoll-Paque PLUS (GE Healthcare).

All the *in vitro* activation assays were performed with cells isolated by FACS (fluorescence-activated cell sorting). For $CD4^+$ and $CD8^+$ T cells sorting, PBMCs isolated from the blood of HDs were treated for 10 minutes with human FcR blocking reagent (Miltenyi Biotec), then stained with mouse anti-human CD4 eFluor 450 (clone OKT4, eBioscience), mouse anti-human-CD8a PE-Cy7 (clone RPA-T8, Biolegend), mouse anti-human-CD56 APC (clone MEM188, eBioscience). Cell-Sorting was performed with a BD FACSAriaTM II Cell Sorter (Flow Core facility, King's College London). $CD4^+$ and $CD8^+$ T lymphocytes were sorted respectively as $CD4^+CD56^-$ and $CD8^+CD56^-$ cells (Figure 3.1). The cells purity was $\geq 99\%$.

For the cytotoxic assays optimization on cancer cells, which were requiring an higher amount of $CD8^+$ cells than what usually obtained by FACS, $CD8^+$ T lymphocytes were isolated with magnetic beads. $CD56^+$ cells were depleted by positive selection using the $CD56^+$ Cell Isolation Kit (Miltenyi Biotec), following manufacturer's instructions. After $CD56^+$ cells depletion, $CD8^+$ T cells were purified with $CD8^+$ Cell Isolation Kit-II (Miltenyi Biotec), following manufacturer's instruction. The purity of cell populations was assessed by flow cytometry co-stain for CD8 and CD56 and it was $\geq 95\%$.

For cell stimulation, 48-well plates were coated overnight at 4°C with 2 μg/ml of anti-CD3 (clone OKT-3), anti-CD28 (BD Biosciences), and anti-CD46 (GB24, generated in house) antibodies; the antibodies were diluted in modified dPBS with calcium and magnesium (Hyclone) to improve their stability. T cells were resuspended and cultured in RPMI 1640 (Gibco), with 1% penicillin-streptomycin and L-glutammine (both from Sigma), and activated in presence of 10U/ml recombinant human IL-2 (a gift from Dr. Pham, Washington University, Saint

Louis). $CD4^+$ and $CD8^+$ cells were plated at a density of $1x10^6$ cells/ml (250 µl cells/well, for a total of $2.5x10^5$ cells/well), and soon after plating cells were spun down for 2 minutes at 500 rpm to allow them to deposit to the bottom of the plate and to bind to the coated antibodies. Primary T cells were left in incubator at 37°C and 5% CO_2, and stimulated for 60 hours.

The NLRP3 inflammasome antagonist MCC950 (Coll *et al*., 2015) was provided by Professor Matthew Cooper (University of Queensland, Australia) and Professor Luke O'Neill (Trinity College, Dublin, Ireland), and used at a concentration of 10 µM in PBS. The C5aR2 agonist P32 (Croker *et al.*, 2016) was provided by Dr. Peter Monk (University of Sheffield, UK) and used at 100 µM, with sterile dH_2O (Hyclone) as vehicle control. Cells were left for 15 minutes in media with the compounds, then directly plated for stimulation.

3.3.2 Flow cytometry analysis

Extracellular stainings were performed in a buffer containing PBS supplemented with 0.5% BSA and 2 mM EDTA (both from Sigma). For intracellular stainings, cells were fixed and permeabilised with Cytofix/Cytoperm kit (BD Biosciences). Before the stainings cells were pre-incubated 10 minutes with human FcR blocking reagent (Miltenyi Biotec). Cells were contemporarily stained for extracellular CD4, CD8 and CD56 expression and gated as shown in Figure 3.1. Stainings were performed with mouse anti-human CD46 PE (clone 8E2, eBioscience), mouse anti-human CD107a FITC (eBioscience), mouse anti-human-granzyme B FITC (BD Pharmigen), mouse anti-human-C5aR1 FITC (clone S5/1, Santa Cruz), mouse anti-human-C5aR2 PE (Biolegend), rabbit anti-human-C5 and goat anti-human NLRP3 (both from Abcam). Secondary antibodies were goat anti-rabbit-PE and donkey anti-goat PE (both from Molecular Probes).

Cleaved caspase-1 staining was performed using the Green FLICA Caspase-1 Assay kit (Immunochemistry Technologies), following manufacturer's instructions.

Data were acquired with a Fortessa LSRIII supported by FACS diva software (BD Biosciences), and analysed by FlowJo 10.0.8 software (Ashland, OR).

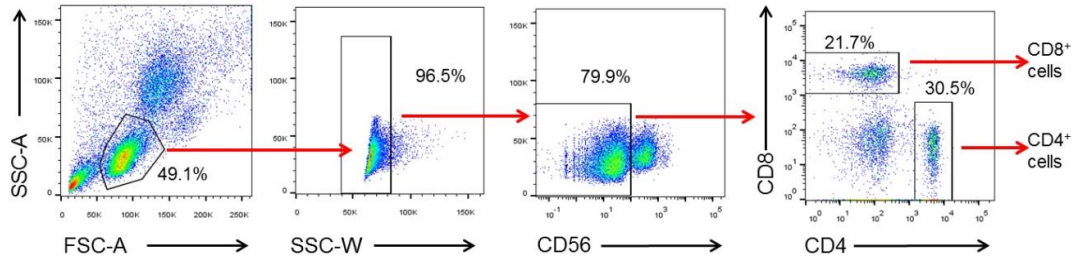


Figure 3.1 Gating strategy used for cell sorting and flow cytometry stainings
Following PBMCs staining, cells were gated accordingly to their size to exclude the debris and myeloid cells, with respectively lower and bigger forward scatter area (FSC-A) and side scatter area (SSC-A) when compared to lymphoid cells. Then doublets of bigger size were excluded for their increased side scatter width (SSC-W) and $CD56^-$ cells were selected accordingly to their expression of CD4 and CD8 for either cell sorting or for identifying cell populations in flow cytometry analysis.

3.3.3 RNA extraction, cDNA synthesis and RT-PCR for CD46 tails

RNA extraction and RT-PCR for CD46 tails were performed by Dr. Gaelle Le Friec, while I prepared resting FACS-sorted $CD4^+CD56^-$ and $CD8^+CD56^-$ cells from 3 HDs fresh blood.

RNA was extracted using the RNeasy Mini kit and the on-column DNAse digestion (both from QIAGEN), following manufacturer's instructions. RNA concentration (A260) and purity (A260/A280 ≈ 2.0) was checked with the spectrophotometer NanoDrop 1000 (Thermo Scientific).

Reverse transcription to cDNA was performed with 200 ng of input RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). The mastermix was generated using 100 pmol random hexamer primers, 1 X reaction buffer, 40 U RiboLock RNase inhibitor, 1 mM dNTP mix, 200 U RevertAid H Minus reverse transcriptase from Moloney Murine Leukemia Virus (M-MuLV) and 100 ng of RNA sample diluted in RNAse-free dH_2O. The reverse transcription reaction was carried out by incubating the samples at 25°C for 5 minutes, followed by a step of 60 minutes at 42°C and a final incubation of 5 minutes at 70°C for enzyme inactivation. Real-Time PCR was performed in 384 wells plate with

7900HT Fast Real-Time PCR System (Thermo Fisher) with the following conditions: 1 X Taqman universal mastermix II with Uracil-DNA Glycosylase (UNG), 100 nM of each primers, 250 nM probe 5' 6-FAM, 3' Iowa Black FQ, 250 nM Eukaryotic 18S rRNA Endogenous Control (VIC®/MGB probe) (all products from Applied Biosystems), 0.5 µl RNase Free water, 2 µl cDNA template (final reaction volume = 10 µl).

CYT1 primers: forward 5'- TTGTCCCGTACAGATATCTTC – 3'

reverse 5'- CTTCTCAGAGAGAAGTAAATTTT - 3'

CYT-1 antisense probe: 5'- AGTTAGGTATGTGCCTTTCTTCTTCCTCC - 3'

CYT2 primers: forward 5'- GGAGGAAGAAGAAAGGGAAAGC - 3'

reverse 5'- TCAGCCTCTCTGCTCTGC - 3'

CYT-2 sense probe: 5'- AGATGGTGGAGCTGAATATGCCACTT - 3'

PCR amplification was performed in an ABI Prism 7900HT thermocycler (Applied Biosystems) for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of combined 15 s at 95°C and 1 min at 60°C. The sequence detector SDS 2.4 software (Applied Biosystems) was used to export the threshold cycles (Ct) values.

Expression of the target gene was normalised using the endogenous gene 18S ribosomal RNA (18S rRNA).

The relative quantification (RQ) of the CYT-1 and CYT-2 expression in $CD8^+$ cells was performed using the ΔΔCt method (Livak and Schmittgen, 2001), where:

$\Delta CT = Ct$ (CYT-1 or CYT-2) – Ct (18S rRNA)

$\Delta\Delta Ct = \Delta Ct$ ($CD8^+$ cells) – (ΔCt $CD4^+$ cells),

$RQ = 2^{-\Delta\Delta Ct}$.

3.3.4 Prostate cancer cells culture and cytotoxicity assay

The primary prostate cancer cell line DU145 (a gift from Dr. Hide Yamamoto, King's College London) was used as APCs source for cytotoxic assay in co-culture with $CD8^+$ cells. Similarly to T cells, DU145 cells were cultured in RPMI 1640 (Gibco), with 1% penicillin-streptomycin and L-glutammine (all from Sigma) and, once confluent, trypsinised with 0.05% trypsin EDTA (Gibco) and passaged to a new flask with fresh media (approximately every 48 hours). The doubling time of DU145

cell line is about 16 hours.

For the cytotoxic assay to measure the cell viability (apoptosis and necrosis), DU145 cells were plated at $1.0x10^4$ cells/well and left to grow in incubator at 37^oC and 5% CO_2, for 24 hours (approximately reaching $2.5x10^4$ cells/well) prior to addition of $2.0\ x10^5$ $CD8^+$ cells on top (~1/8 ratio).

Beads-sorted $CD8^+CD56^-$ cells were marked with carboxyfluorescein succinimidyl ester (CFSE) to further distinguish them from the DU145 cells in the co-culture. $CD8^+$ cells were stained at a density of $1x10^7$ cells/mL in PBS, at room temperature (RT), with 2.5 μM of CFSE (Thermo Fisher) for 2 minutes, and 5 volumes of culture media were added to wash the unbound CFSE. Cells were then resuspended in fresh media at $1x10^6$ cells/mL and activated as described above (section 3.3.1) in 48 well plates for 24 hours. Following activation, T cells were harvested and added on top of the DU145 cells, the 48 well plate was spun at 500 rpm for 2 minutes to allow the $CD8^+$ cells to deposit to the bottom in co-culture with the cancer cells, and the plate was left to incubate for other 12 hours.

After this overnight incubation, the co-culture of DU145/$CD8^+$ cells was harvested from the single wells into separate eppendorf tubes, the media was washed out by 5 minutes centrifugation at 300g, at RT, the supernatants were collected to check for cytokine production. PBS was added immediately after cell collection for 1 minute to wash the wells in the 48 well plate and remove the rest of media from the DU145 cells still adhering to the plate. After PBS removal, detachment of the adherent cells was performed by addition of 100 μl/well of 0.05% trypsin EDTA (Gibco) for 2 minutes, then the detached cells were collected in the corresponding eppendorf tubes where non-adherent cells were previously collected.

Trypsin was removed by centrigugation at 300 g for 5 minutes at RT, then the cells were resuspended in 100 μl of Annexin V binding buffer (BD Biosciences) and transferred to minitubes for flow cytometry. Annexin V staining was carried out at RT for 15 minutes with 3 microL/test of Annexin V-APC, following addition of 0.5 μg/ml propidium iodide (PI) (both from BD Biosciences) 2 minutes before flow cytometry acquisition. Cells were analysed on a FACS Calibur cytometer, supported by CellQuest Pro software (both from BD Biosciences). The DU145 cells were gated as FL1-H negative (no green stain), because $CD8^+$ cells were marked with the green CFSE dye. Annexin V single positive cells, Annexin V/PI double positive cells and

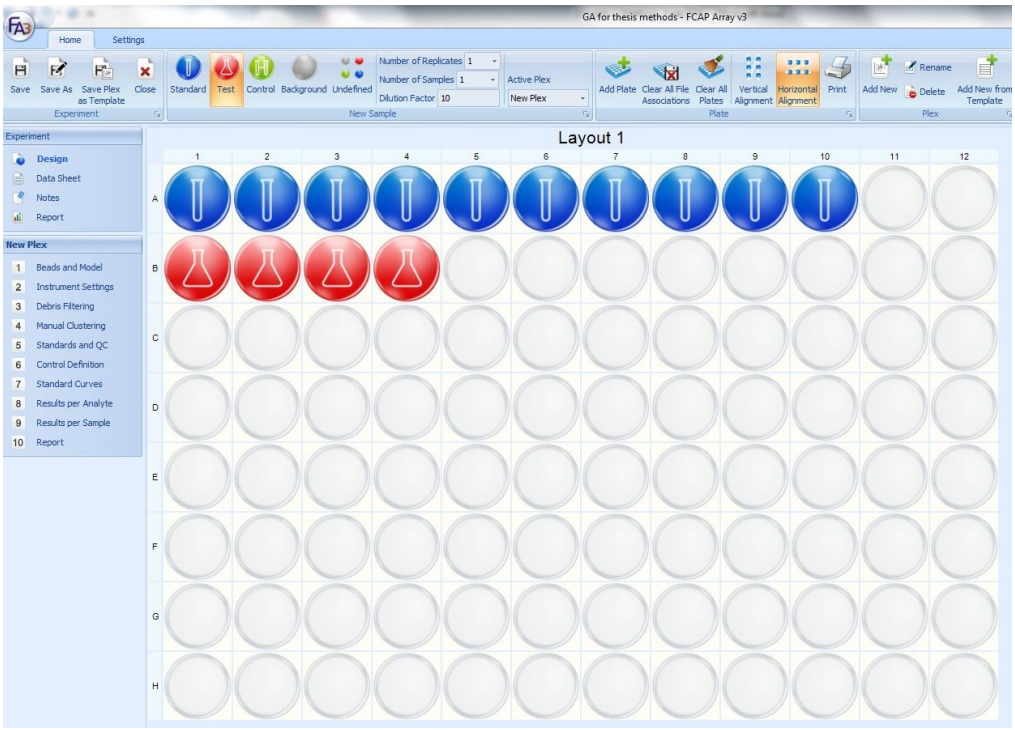
PI single positive cells were considered respectively as early apoptotic, late apoptotic and necrotic.

3.3.5 Cytokine measurement

Cytokine production was quantified from cell supernatants using the human Th1/Th2/Th17 Cytometric Bead Array (BD Biosciences) with the respective preparation of cytokines standard, following the manufacturer's instructions, and samples were acquired using the 96 well plate automate reader system on a Fortessa LSRIII supported by FACS diva software (BD Biosciences). Data were analysed by FCAP array software (Soft Flow Hungary Ltd). The analysis was performed by following these steps (Figure 3.2):

a) generation of a template plate, to export the single flow cytometry files (.fcs) acquired by the Fortessa LSRIII, and assignment of samples dilution factors (1/10 to 1/25 dilution of supernatants was used) and of the kit used, the Human Th1/Th2/Th17 Cytokine kit - 560484;
b) assignment of correspondent cytokine to each beads histogram peak of APC-A (following manufacturer's instructions) and debris filtering;
c) assignment of standard curve, with higher standard of 5000 pg/ml cytokine, serial 1/2 standards dilutions and lower standard at 0 pg/ml concentration (blank);
d) generation of the standard curve and individual analysis of each cytokine per sample.

A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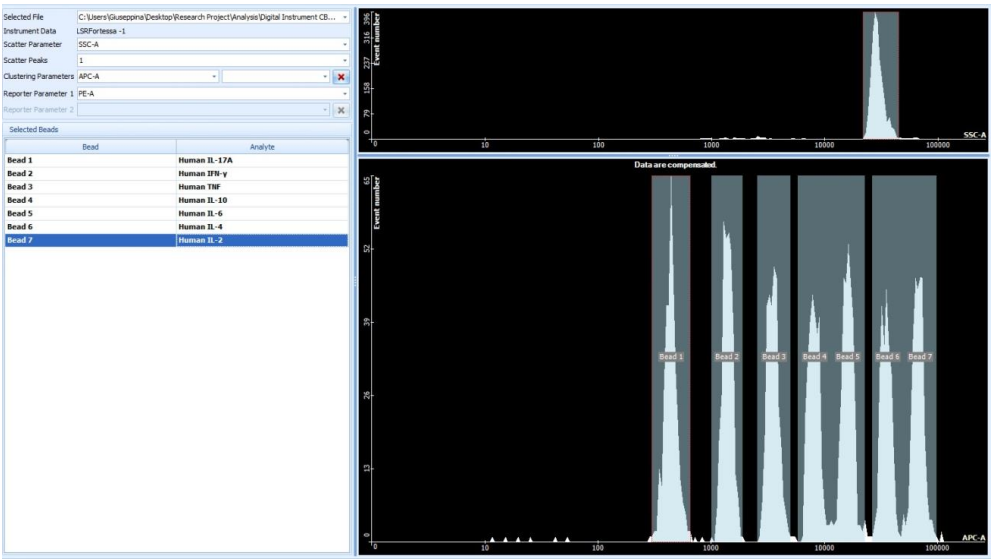
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Bead 3 Human TNF
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Bead 5 Human IL-6
Bead 6 Human IL-4
Bead 7 Human IL-2
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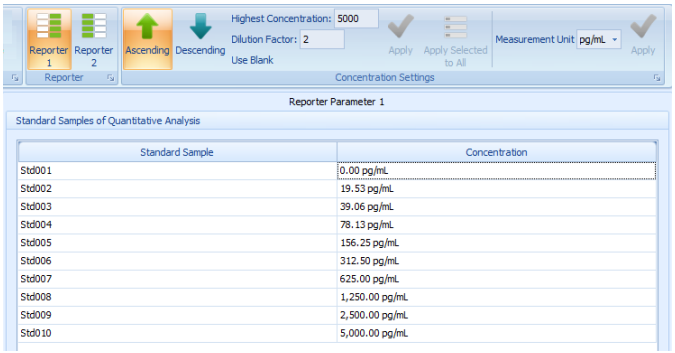
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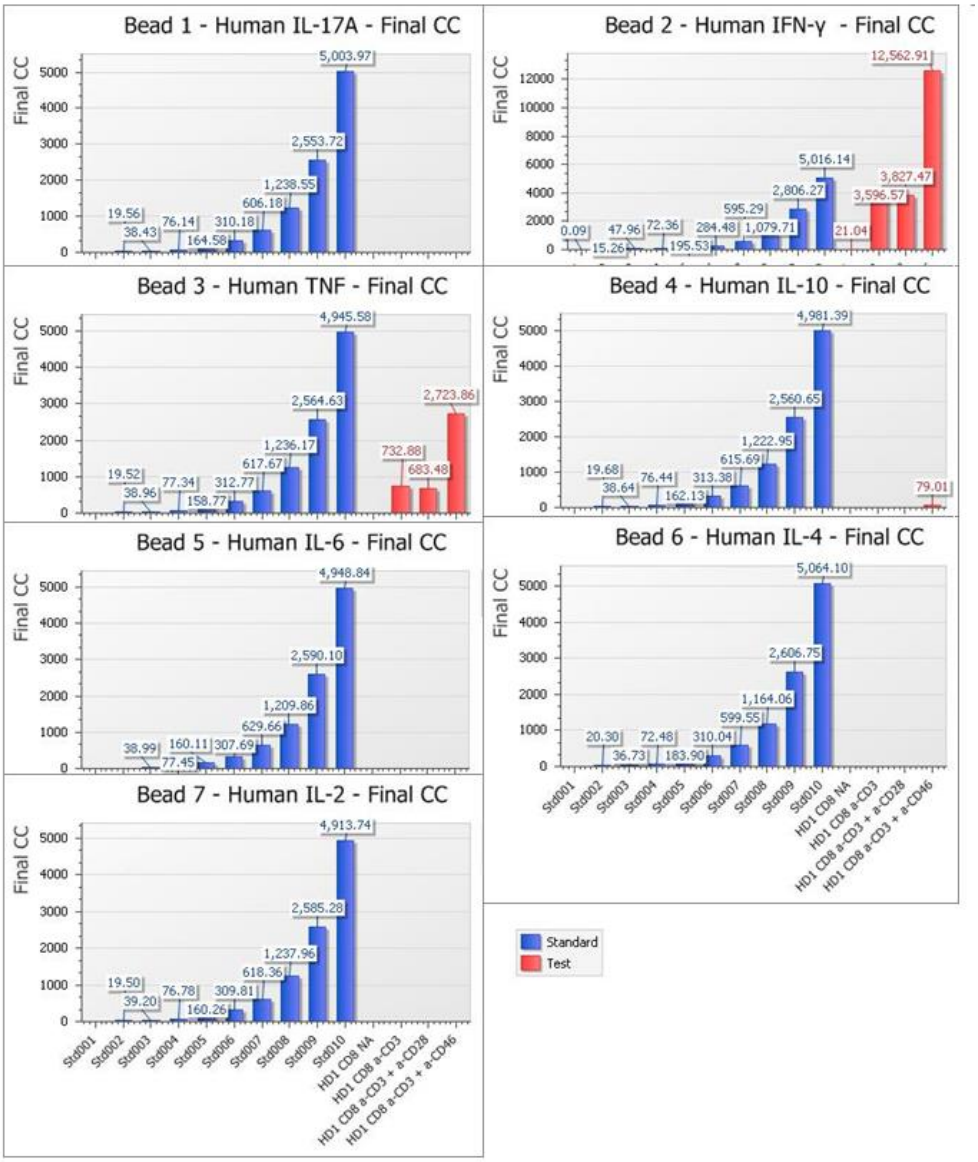


Figure 3.2 Cytometric bead array analysis using the FCAP software
(a) Graphical interface of the software, where single .fcs files can be exported onto a graphical plate (standards reported in blue and samples in red), dilutions can be assigned, then the user can follow the 10 steps reported on the left window, starting from the step 1 of assignment of the beads model use (in this case the Human Th1/Th2/Th17 Cytokine kit – 560484); (b) step 2, the "Instrument settings", where beads are assigned to corresponding cytokines accordingly to the APC-A, then this is followed by debris filtering accordingly to the FSC-A, and in some cases by manual clustering; (c) standard command, where lower and upper standards value are reported, the order (ascending or descending) and dilutions factors are given; (d) the step 8, "result per analyte", in this case for the standards 0 to 5000 ng/mL plus an example of HD $CD8^{+}CD56^{-}$ cells either left non activated (NA) or in vitro activated with anti-CD3, anti-CD3 + anti-CD28 or anti-CD3 + anti-CD46 antibodies. It is evident that $CD8^{+}$ cells produce only a substantial level of IFN-γ and TNF-α and a minimal amount of IL-10, while other cytokines are not detectable.

3.3.6 Confocal microscopy

Sorted cells $CD4^+CD56^-$ and $CD8^+CD56^-$ from HDs PBMCs, either left non activated or *in vitro* pre-activated for 60 hours with anti-CD3 + anti-CD46, were fixed, permeabilised and stained in the same way as for the flow cytometry intracellular staining procedure showed above. NLRP3 staining was performed using the primary antibody goat anti-human NLRP3 (Abcam) and the secondary antibody donkey anti-goat Alexa fluor 488 (Abcam). Cells were mounted using VECTASHIELD media with DAPI (Vector labs). Images were acquired with a Nikon A1R confocal microscope (Nikon Imaging Centre, KCL) using 100X oil immersion objective, and analysed by NIS Elements software (Nikon).

3.3.7 Data analysis

Statistics analyses were performed using the one-way ANOVA or the paired *t*-test and Bonferroni correction where appropriate (GraphPad Prism software). Data were assessed for normal distribution using the D'Agostino-Pearson test. Data were expressed as mean ± standard error of the mean (SEM), and statistical significance was attributed to p values < 0.05.

3.4 Results

In principle, an approach similar to the one adopted in studying $CD4^+$ T cells has been implemented for testing the initial hypotheses, in such that it has been firstly assessed if the complement receptors and components in questions and the NLRP3 inflammasome components are present in human $CD8^+$ T cells and whether they are affected by cell activation. The cytokines production and cytotoxic activity of cells were assessed upon TCR and CD46 stimulation in presence or absence of an agonist modulating the activity of the surface C5aR2 or the inflammasome specific inhibitor MCC950. *In vivo* significance of the observations herein reported would then be planned by utilising $CD8^+$ T cells from CAPS patients and from *Nlrp3* and *Il1b* and *Il1r* deficient animals in suitable *in vivo* models.

Below it is listed the set of experiments that I have been able to perform so far with a discussion on the strengths and shortcomings of the data, their implications for the role of the autocrine/intracellular complement and the NLRP3 inflammasome in $CD8^+$ T cell responses and an outlook into potential future follow-up studies.

3.4.1 Effects of CD46 *in vitro* co-stimulation on $CD8^+$ T cell cytokine production and granzyme B expression/degranulation

Before starting *in vitro* functional studies on human $CD8^+$ T lymphocytes, a thorough analysis of CD46 expression was performed in human $CD4^+$ versus $CD8^+$ T lymphocyte subsets sorted by flow cytometry from the fresh blood of healthy volunteers. Cells were also sorted for lack of CD56 expression, which is an NK cell marker ($CD56^-$), to exclude the contamination with NK or NKT cellular subsets (Bendelac et al., 2007). Flow cytometry staining demonstrated that, in line with published literature, $CD8^+$ T cells express CD46 and that total CD46 protein expression levels are not significantly different between the two T cell subsets - although there is a trend towards less CD46 protein expression in $CD8^+$ cells versus $CD4^+$ T cells (Figure 3.3A). Because the CYT-1-bearing isoforms of CD46 drive IFN-γ production in $CD4^+$ T cells, the CD46 tails expression pattern was assessed in freshly isolated resting $CD8^+$ T cells. These experiments suggested that there is a

trend in $CD8^+$ T lymphocytes towards higher mRNA expression for the CD46 isoforms bearing the CYT-1 and CYT-2 tails, evident in 2 out of 3 healthy donors used (and normalised to $CD4^+$ T cells from the same donor). Interestingly, a third donor showed much lower CD46-CYT-1 and CD46-CYT-2 mRNA expression in this cell subset when normalised to expression levels in $CD4^+$ cells (Figure 3.3B). Noteworthy, this reduced expression did not affect *in vitro* cytokine production by $CD8^+$ lymphocytes from this donor (data not shown). Overall, CD46 is expressed also by $CD8^+$ T cells, without substantial differences for CYT-1 and CYT-2 tails transcripts levels.

Next, the effect of CD46 co-stimulation during $CD8^+$ T lymphocyte activation on cytokine production and on granzyme B degranulation (which is a key part of their cytotoxic function) was analysed *in vitro*. To this end, $CD4^+CD56^-$ and $CD8^+CD56^-$ cells, isolated from the same HDs by FACS sorting (purity $\geq$ 99%), were activated with anti-CD3 antibody alone or in combination with either anti-CD28 or anti-CD46 antibodies. Both cytokine production and degranulation were analysed after 60h of *in vitro* activation.

In analogy to $CD4^+$ T cells, $CD8^+$ T cells respond to CD46 co-stimulation with significantly increased IFN-γ and TNF-α production. However $CD8^+$ lymphocytes do not respond to CD46 co-stimulation with an increased switch into an IL-10-producing phenotype as IL-10 secretion by these cells is in the order of pg/ml and with an average IFN-γ to IL-10 ratio of 100 in $CD8^+$ cells versus 1.5 in $CD4^+$ cells (Figure 3.4). Secretion of IL-2, IL-4, IL-6 and IL-17 was barely detectable from $CD8^+$ cells (data not shown). Importantly, CD28 co-stimulation does not affect production of these cytokines when compared to the stimulation of TCR alone with immobilised anti-CD3 antibody.

Similarly to its effect on cytokines production, CD46 *in vitro* co-stimulation of $CD8^+$ T cells induces significantly increased degranulation when compared to anti-CD3 and anti-CD3 + anti-CD28 co-stimulation, as demonstrated by up-regulation of extracellular CD107a and intracellular content of granzyme B (Figure 3.5).

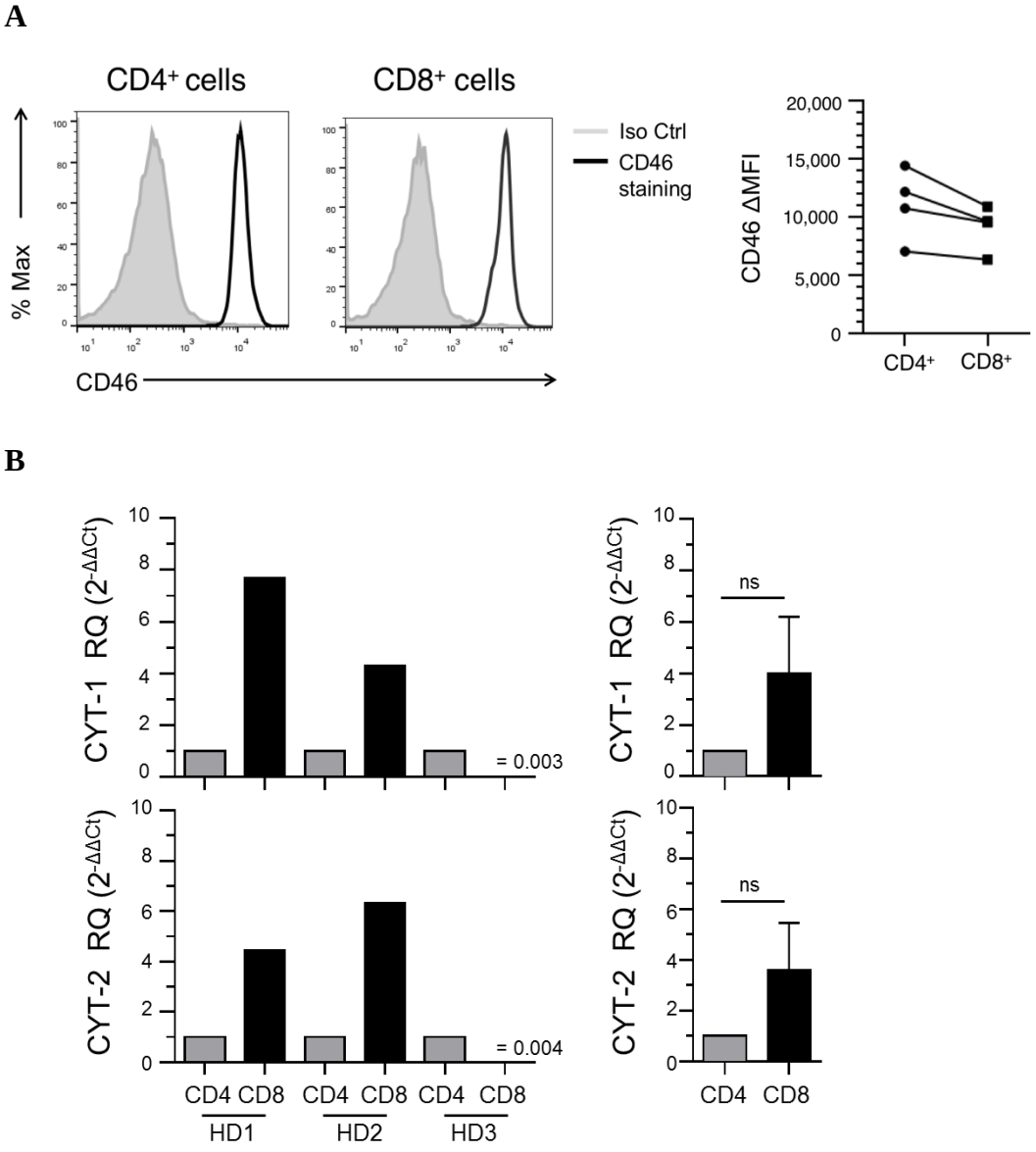


Figure 3.3 Analysis of CD46 protein and mRNA coding for CYT-1 versus CYT-2 tails in CD8$^+$ T lymphocytes

(a) Flow cytometry analysis of CD46 total protein expression in CD4$^+$ and CD8$^+$ T lymphocytes subsets (CD56$^-$ to exclude NK cells) from freshly isolated PBMCs, with relative variation in the geometric mean fluorescence intensity (ΔMFI) compared to the isotype control (n=4). (b) RT-PCR for the CD46 tails CYT-1 and CYT-2 in CD8$^+$ versus CD4$^+$ T lymphocytes sorted from HDs fresh blood, with relative quantification (RQ) showed as individual values for 3 healthy donors or together in bar graphs (average of 3 technical replicates of n=3 biological replicates): $RQ = 2^{-\Delta\Delta Ct}$, with $\Delta\Delta Ct = \Delta Ct$ (CD8$^+$ cells) – (ΔCt CD4$^+$ cells), $\Delta CT = Ct$ (CYT-1 or CYT-2) – Ct (RNA 18S), Ct = threshold cycles. The RT-PCR experiments under (b) were performed by Dr. Gaelle Le Friec.

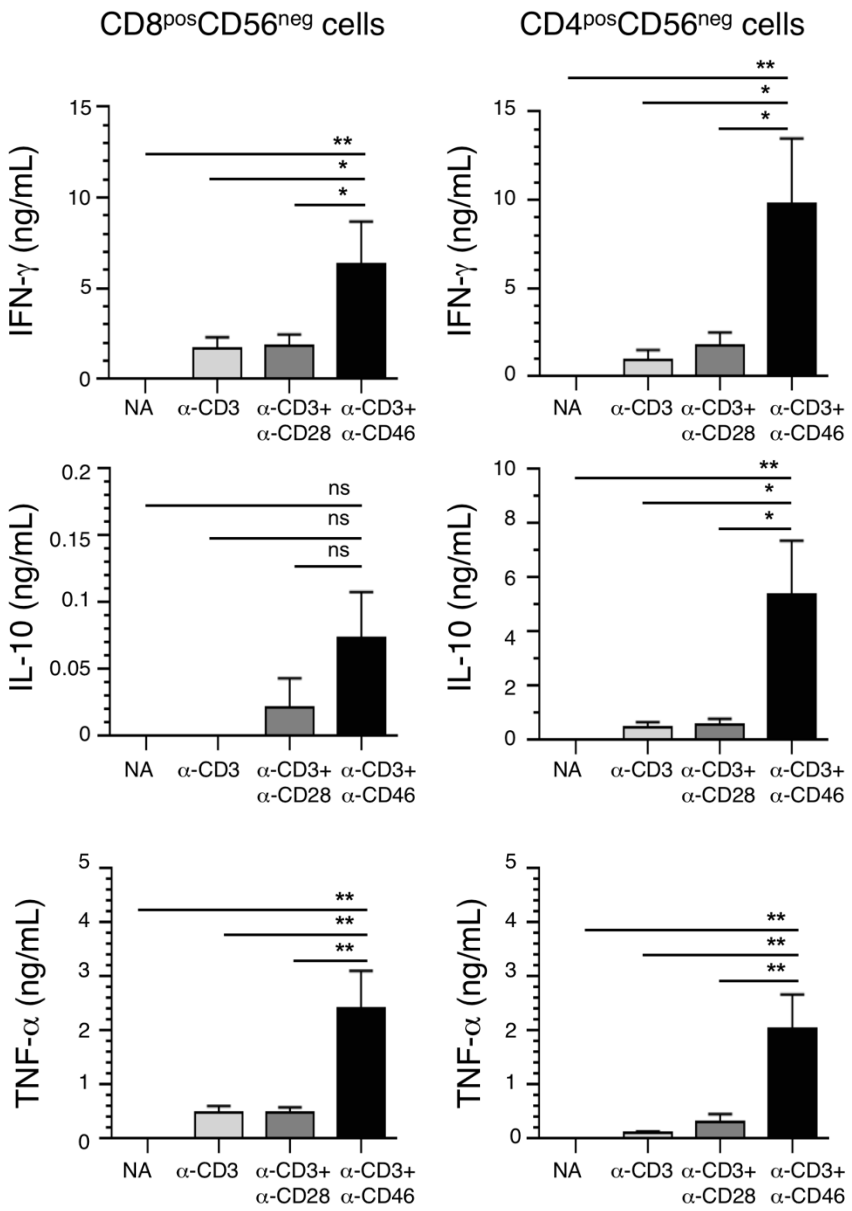


Figure 3.4 CD8$^+$ T lymphocytes cytokine secretion upon CD46 co-stimulation
Secreted IFN-γ, IL-10 and TNF-α (ng/mL) by CD8$^+$CD56$^-$ versus CD4$^+$CD56$^-$ T lymphocytes from the same donors, where FACS-sorted cells from the blood of HDs, left either non-activated (NA) or activated *in vitro* (60 hours) with anti-CD3 (α-CD3), α-CD3 + α-CD28, or α-CD3 + α-CD46. Data are represented as means ± standard error of the mean (SEM), n=5. *P < 0.05, **P < 0.01, ns = not significant, one-way ANOVA with Tukey multiple comparison test.

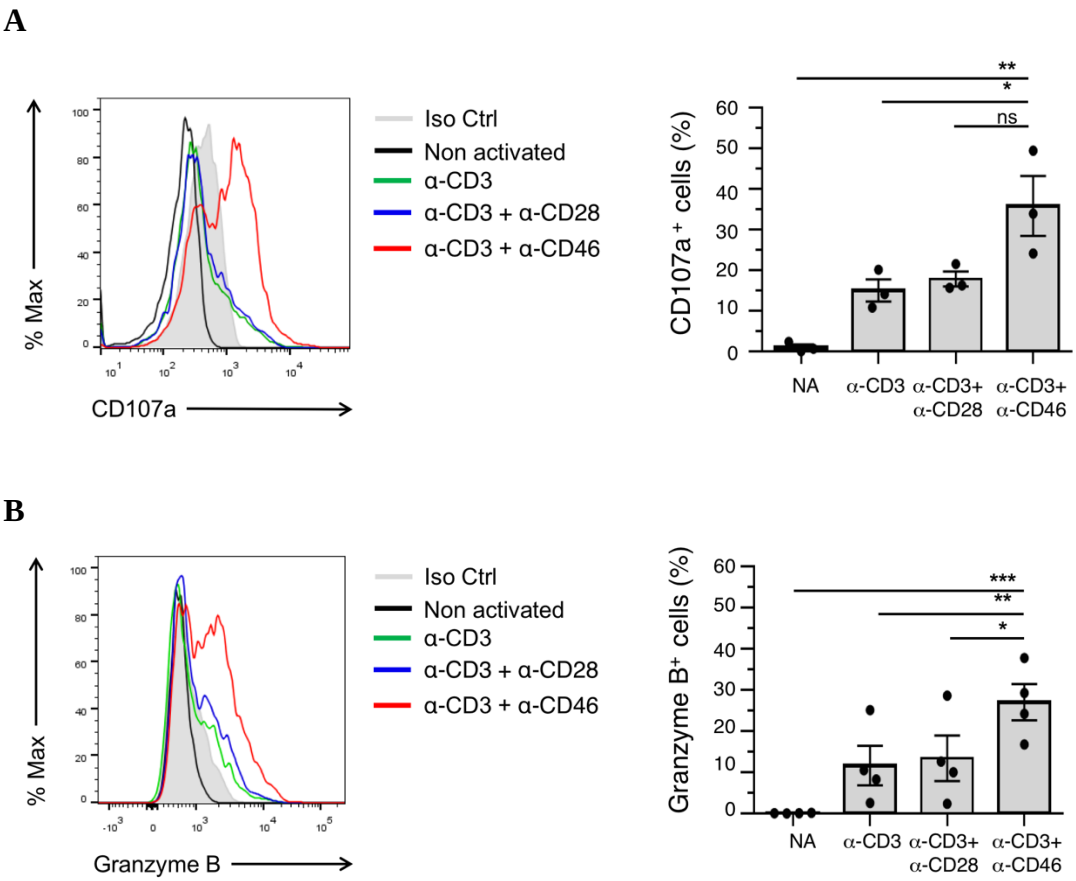


Figure 3.5 CD8$^+$ T lymphocytes degranulation upon CD46 co-stimulation
Flow cytometry staining for surface CD107a (a) and intracellular granzyme B (b) on CD8$^+$CD56$^-$ FACS-sorted cells isolated from the blood of HDs, left non activated or activated *in vitro* (60 hours) with anti-CD3 (α-CD3), α-CD3 + α-CD28, or α-CD3 + α-CD46, in presence of 10U/mL IL-2. Data are represented as means ± SEM, n=3 (a) and n=4 (b). *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant, one-way ANOVA with Tukey multiple comparison test.

3.4.2 Effect of CD46 co-stimulation on $CD8^+$ T cell cytotoxicity

Having observed that CD46 co stimulation of $CD8^+$ T cells induces increased IFN-γ production and granzyme B expression and cell degranulation, the next step was to test if this possibly translates into increased cytotoxicity of these cells towards cancer cell killing. For this, a cytotoxicity assay involving activated $CD8^+$ T cells and the prostate cancer cells DU145 was established.

As CD46 is expressed ubiquitously in all human nucleated cells (Yamamoto *et al*., 2013), to analyse the specific effect of CD46 antibody-mediated stimulation on $CD8^+$ T lymphocytes, excluding any direct effect of stimulation of surface CD46 expressed by the prostate cancer cells, a cytotoxicity assay was set by transferring pre-activated $CD8^+$ cells onto DU145 cells cultured in 48-well plates. Further, to distinguish epithelial cancer cells from blasting activated $CD8^+$ T cells, whose size does overlap in terms of forward scatter (FSC) and side scatter (SSC) in FACS analyses, $CD8^+$ lymphocytes were first stained with a cell permeable not toxic dye (CSFE) before undergoing *in vitro* activation. Thus, DU145 cells were distinguished from $CD8^+$ T cells after co-culture for their bigger size and the lack of the green CSFE staining. Once co-incubated with pre-activated $CD8^+$ cells for 12 hours, DU145 cells were stained for the apoptosis marker Annexin V, which is upregulated on the cell surface of apoptotic cells (Vermes *et al*., 1995), and the necrosis marker propidium iodide (PI, which is cell impermeable and enters only into dying cells with loss of cytoplasmic membrane integrity). $CD8^+$ T lymphocytes pre-activated with CD46 co-stimulation demonstrated an increased ability to kill DU145 cells, with a 21.2 % versus 10.42 % of late apoptotic cells and 22.6% versus 20.8% necrotic cells when compared with the CD28 co-stimulation (Figure 3.6A). To monitor for proper CD46 co-stimulation, IFN-γ production was also measured in cultures and was increased in those containing α-CD3 + α-CD46 pre-activated $CD8^+$ T cells (Figure 3.6B).

These experiments need to be repeated with T cells from different donors (thus allowing to perform statistical analyses), but this assay may be used in future to investigate the mechanisms of CD46-mediated $CD8^+$ T cell cytotoxicity and the role of specific molecules (using specific agonists and/or inhibitors) in this process.

In sum, CD46 appears to provide a much more potent co-stimulatory signal compared to CD28 for *in vitro* activation of human $CD8^+$ T lymphocytes and their effector functions including IFN-γ and TNF-α cytokine production, cytotoxic granules release and cytotoxic activity at least towards a cancer cell line. However, different from human $CD4^+$ T cells, CD46 co-stimulation does not induce substantial levels of IL-10 (switching) in $CD8^+$ T cells.

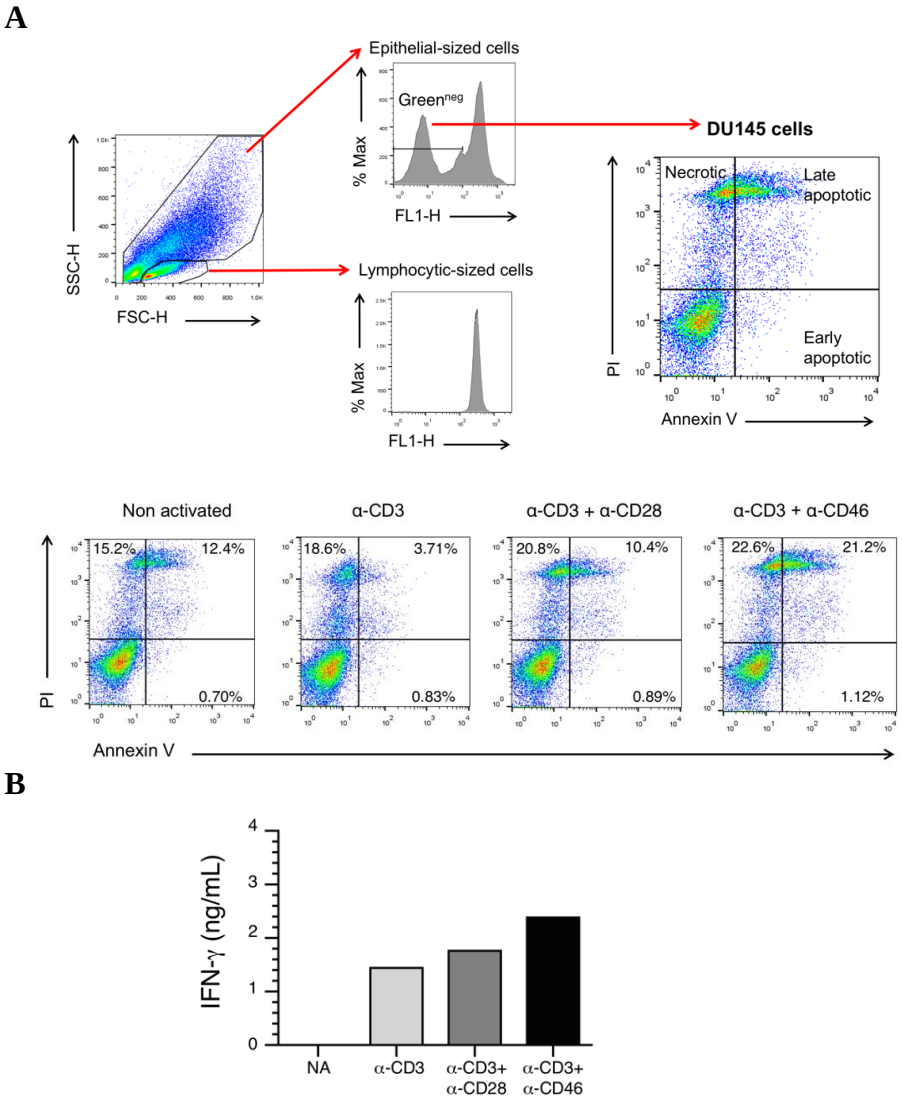


Figure 3.6 Effect of CD46 co-stimulation on $CD8^+$ T cell-mediated killing of DU145 cells. $1.0x10^4$ DU145 cells ($5.0x10^4$ cells/mL) were cultured in 48 well plates and left to adhere and proliferate. $CD8^+CD56^-$ cells, bead-isolated from HDs fresh blood and stained with a green dye (CSFE), were left either non-activated or were activated *in vitro*, as described under Fig. 3.4, with α-CD3, α-CD3 + α-CD28 or α-CD3 + α-CD46 antibodies for 24 hrs. These pre-activated $CD8^+$ T cells were then added on top of the plated DU145 cells at approximately 1/8 ratio ($2.0x10^5$ T cells on top of ~$2.5x10^4$ DU145 cells). (a) Cells were stained for Annexin V (apoptosis marker) and PI (necrosis marker). The cell mixtures were cultured for another 12 hrs and the killing of the CSFE-negative DU145 target cells by the CSFE-positive $CD8^+$ T cells was assessed using the annexin V and PI staining. The top panel shows the gating strategy: the cancer epithelial cells were selected accordingly to the bigger size (in term of FSC-H and SSC-H), green positive cells (bigger blasting T lymphocytes) were excluded, then cells were gated as early apoptotic (Annexin V^{pos}, PI^{neg}), late apoptotic (Annexin V^{pos}, PI^{pos}) and necrotic (Annexin V^{neg}, PI^{pos}). The bottom panel shows the dot plots for the Annexin V and PI co-staining for DU145 cells gated as on top panel. (b) Pre-activation of $CD8^+$ T cells was monitored by measuring secreted IFN-γ (ng/ml) by the $CD8^+CD56^-$ after 24h of activation.

3.4.3 Investigating a C5 complement system in $CD8^+$ T lymphocytes

After clearly demonstrating an intrinsic C3 and C5 complement system in $CD4^+$ T lymphocytes and its role in modulating Th1 responses, and showing that $CD8^+$ T cells also respond to CD46 activation (hence C3-derived signals), preliminary experiments were performed to investigate whether a 'C5 system' may also exist in $CD8^+$ T lymphocytes and its possible role in regulating cell activation and effector cytotoxic functions. In the same way as for $CD4^+$ cells, also $CD8^+$ T lymphocytes demonstrate an exclusive intracytoplasmic C5aR1 protein expression and both extracellular and intracellular localization for C5aR2 (Figure 3.7). Moreover, $CD8^+CD56^-$ T lymphocytes display total levels of the complement component C5 and the C5aR1 and C5aR2 comparable to the expression level in $CD4^+CD56^-$ T lymphocytes from the same HDs PMBCs (Figure 3.8). An outstanding experiment that still needs to be performed is the measurement of whether activated $CD8^+$ T cells generate intracellular C5a.

Because surface-expressed C5aR2 has been shown to negatively regulate Th1 responses (Chapter 2), a possible effect of C5aR2 agonism, using the same specific C5aR2 peptide agonist tested on human $CD4^+$ T cells, was studied in $CD8^+$ T cells. Indeed, following *in vitro* activation with anti-CD3 + anti-CD46 antibodies, $CD8^+CD56^-$ FACS-sorted lymphocytes treated with the C5aR2 agonist showed significantly reduced IFN-γ secretion without significant effects on IL-10 and TNF-α production, although there is a trend towards augmented IL-10 and reduced TNF-α (Figure 3.9). This reduction in cytokine production is also accompanied by a similar effect on cytotoxic granule release, as demonstrated by decreased surface CD107a and intracellular granzyme B in $CD8^+$ T cells activated in presence of C5aR2 agonism (Figure 3.10). Of note, FSC versus SSC analyses of FACS plots suggest that cell viability is not affected by C5aR2 agonism in $CD8^+$ T cells (data not shown).

Overall, these data demonstrate (similar to $CD4^+$ T cells) the existence of a "C5 system" also in human $CD8^+$ T lymphocytes, and further support a functional role for C5aR2 in the negative regulation of $CD8^+$ T cell effector functions.

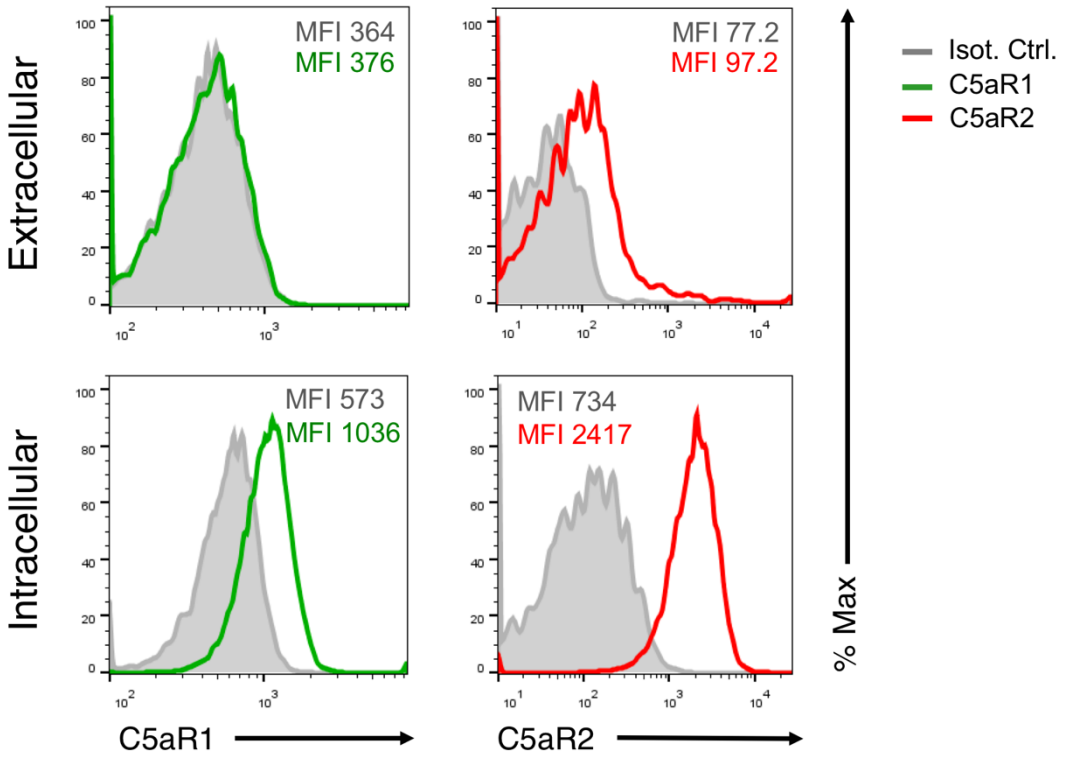


Figure 3.7 C5aRs expression by human CD8$^+$ T lymphocytes
Flow cytometry analysis of extracellular and intracellular (total) protein expression for C5aR1 and C5aR2 on resting CD8$^+$CD56$^-$ T lymphocytes isolated from HDs PBMCs, with histogram plots (representative of n=3 experiments), showing the correspondent geometric mean fluorescence intensity (MFI).

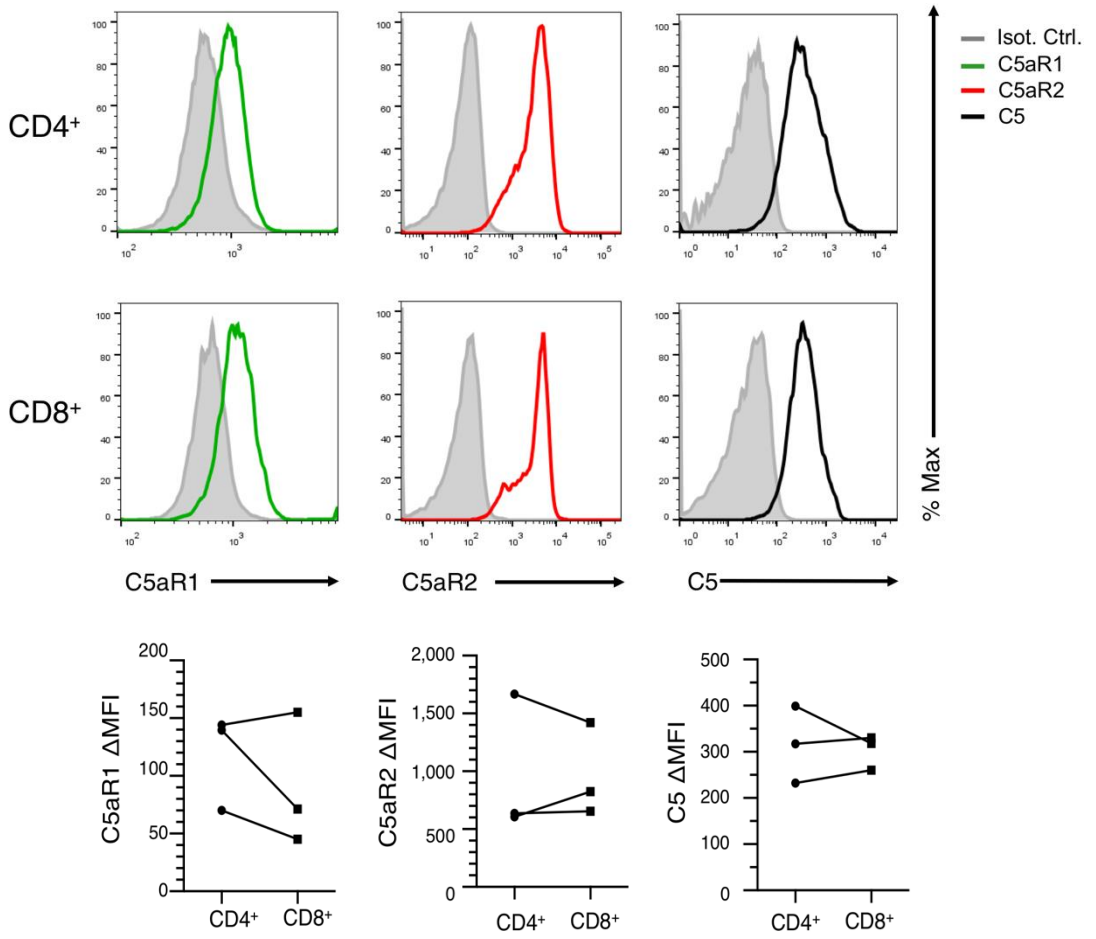


Figure 3.8 C5aR1, C5aR2 and C5 expression in CD8$^+$ versus CD4$^+$ T cells
Flow cytometry dot plots representative of C5aR1, C5aR2 and C5 total (intracellular) protein expression in CD4$^+$ and CD8$^+$ T lymphocytes (CD56$^-$ to exclude NK cells) from HDs fresh blood isolated PBMCs, with the respective variation in the geometric mean fluorescence intensity (ΔMFI) to the isotype control (n=3).

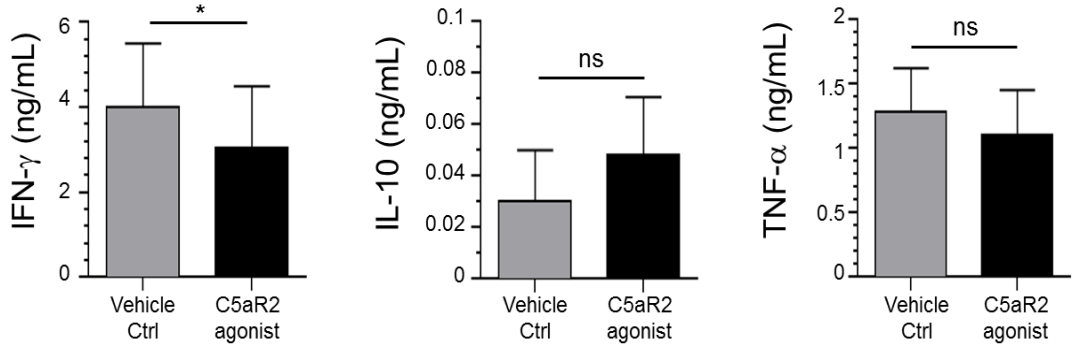
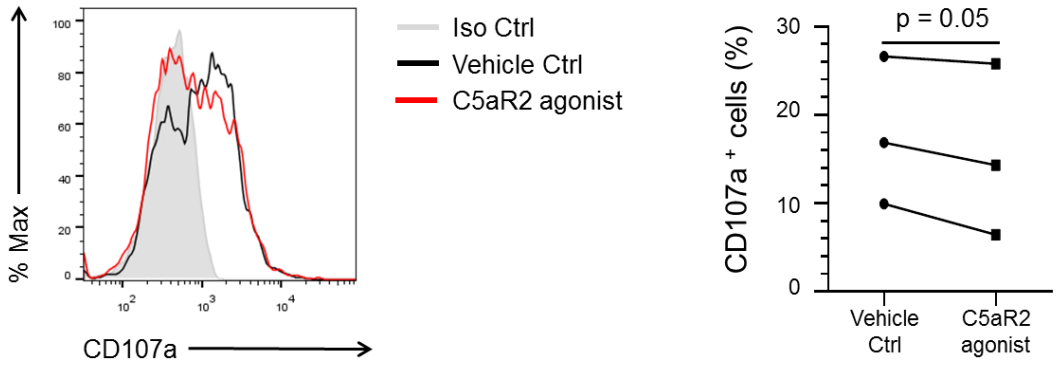


Figure 3.9 Effect of C5aR2 agonism on *in vitro* cytokine secretion by CD8$^+$ T cells

Secreted IFN-γ, IL-10 and TNF-α (ng/mL) by CD8$^+$CD56$^-$ FACS-sorted cells from HDs and activated *in vitro* (60 hours) with α-CD3 + α-CD46, in presence of C5aR2 peptide agonist (100 μM) or vehicle control. Data are represented as means ± SEM, n=6. *P < 0.05, ns = not significant, paired Student's *t*-test.

A



B

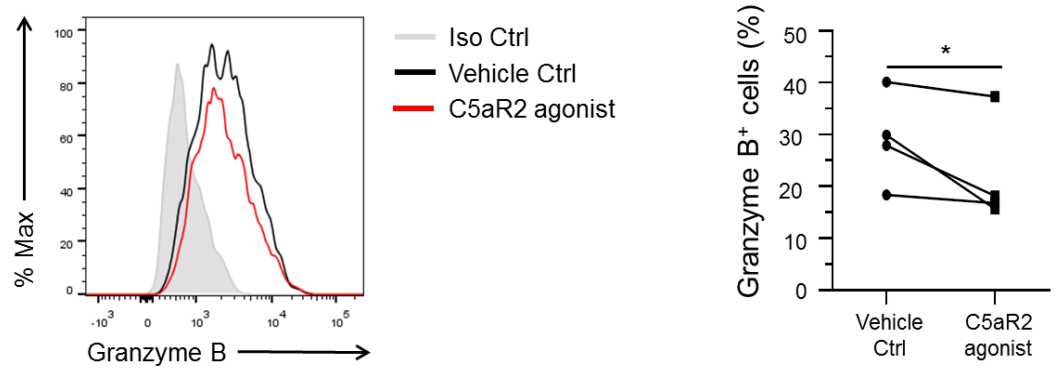


Figure 3.10 Effect of C5aR2 agonism on *in vitro* degranulation and granzyme B expression by CD8$^+$ T cells

Flow cytometry staining for surface CD107a (a) and intracellular granzyme B (b) on CD8$^+$CD56$^-$ FACS-sorted cells from HDs fresh blood, activated in vitro (60 hours) with α-CD3 + α-CD46, in presence of C5aR2 peptide agonist (100 μM) or vehicle control. Data are represented as means ± SEM, n=3 (a) and n=4 (b). *P < 0.05, paired Student's *t*-test.

3.4.4 NLRP3 expression and modulation in $CD8^+$ T lymphocytes

Having established that human $CD8^+$ T cells seem to contain a similar autocrine C3 and C5 'machinery' as observed in human $CD4^+$ T cells, the next set of experiments then aimed at addressing whether $CD8^+$ lymphocytes can also assemble a functional NLRP3 inflammasome and whether this intrinsic inflammasome may contribute to IFN-γ secretion (or even cytotoxicity). Indeed, the NLRP3 protein is present also in $CD8^+CD56^-$ T cells, as shown by techniques including intracellular immunostaining, using flow cytometry and confocal microscopy (Fig. 3.11). Levels of expression of the NLRP3 protein appear similar in resting and *in vitro* activated $CD4^+$ and $CD8^+$ cells (Fig. 3.11A and B). Moreover, activated $CD8^+$ T lymphocytes, particularly upon CD46 co-stimulation, show up-regulated NLRP3 protein (Fig. 3.11C). Therefore, this study proceeded with investigating whether a canonical NLRP3 inflammasome assembles in $CD8^+$ T lymphocytes. Very surprisingly though, and in contrast to observations in human $CD4^+$ T cells, when $CD8^+CD56^-$ cells FACS-sorted from HDs were activated *in vitro* in presence of the specific NLRP3 inflammasome antagonist MCC950 (Coll *et al*., 2015), no effects were observed on IFN-γ, IL-10 and TNF-α cytokine production (Fig. 3.12), caspase-1 cleavage (Fig. 3.13A) or granzyme B production (Fig. 3.13B). This was unexpected (a reduction at minimum in IFN-γ secretion was anticipated) and suggests that either 1) intrinsic assembly of a functional canonical NLRP3 inflammasome might not occur in $CD8^+$ cytotoxic T cells or, 2) if present, optimal/normal IFN-γ production by $CD8^+$ T cells is not dependent on intrinsic NLRP3-inflammsome driven IL-1β secretion. This latter idea is supported by the fact that both IL-1β and IL-18 cytokines secretion was not detectable by $CD8^+$ cells (data not shown).

In sum, human $CD8^+$ T cells clearly express NLRP3 and can generate active caspase-1 but neither activity seems to be required for optimal IFN-γ secretion in these cells – which is in contrast to observations in human $CD4^+$ T cells.

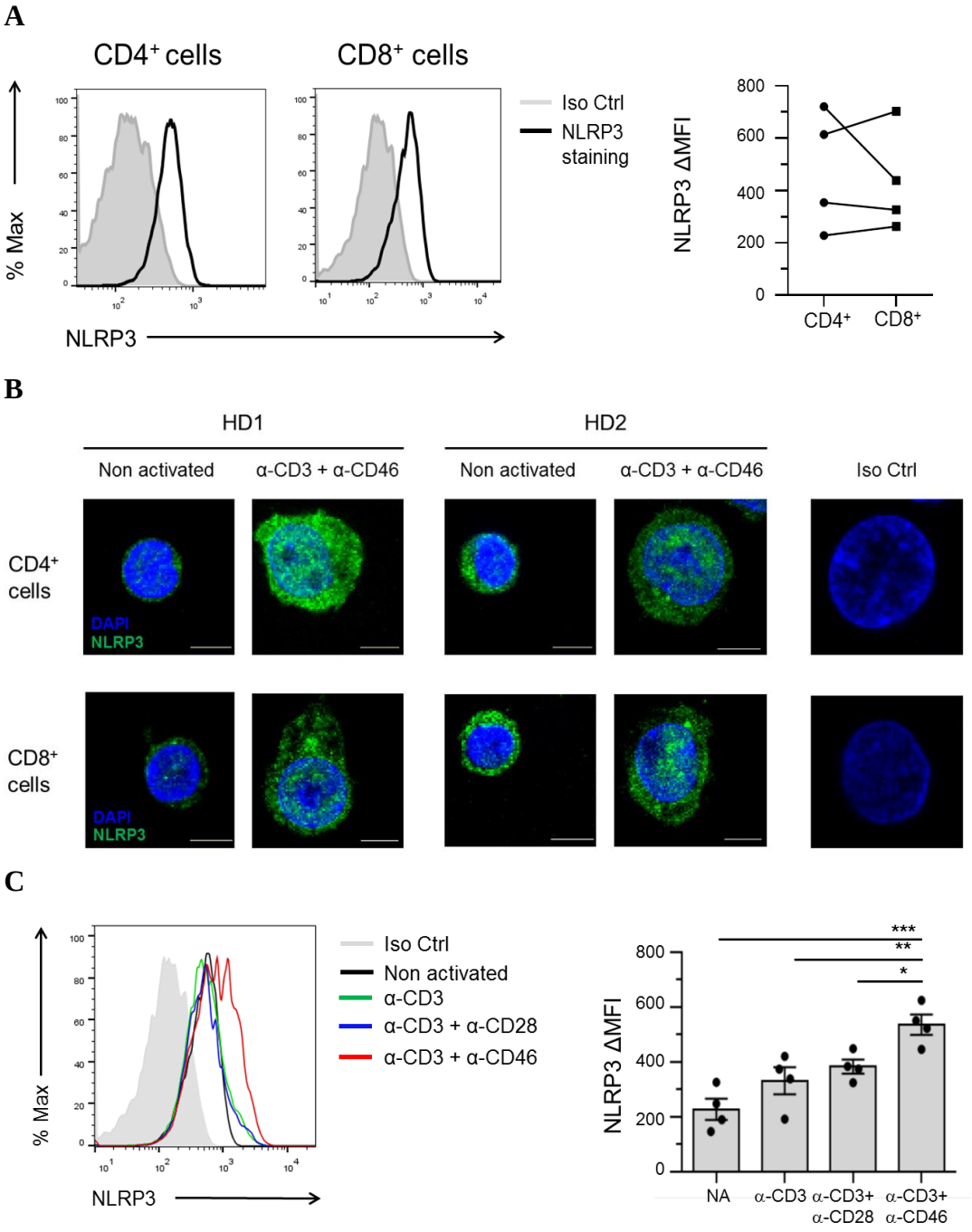


Figure 3.11 NLRP3 protein expression by resting and activated CD8$^+$ T cells
(a) Flow cytometric analysis of NLRP3 protein expression in CD4$^+$ and CD8$^+$ T lymphocytes isolated from HDs, with relative variation in the geometric mean fluorescence intensity (ΔMFI) to the isotype control (n=4). (b) NLRP3 confocal staining (green) in resting and α-CD3 + α-CD46 activated CD4$^+$ and CD8$^+$ cells from 2 HDs, nuclei in DAPI, scale bar = 10 µm. (c) Flow cytometry histogram plots for intracellular NLRP3 protein content in CD8$^+$CD56$^-$ FACS-sorted cells from HDs (n=4), left either non-activated or activated *in vitro* (60 hours) with α-CD3, α-CD3 + α-CD28, or α-CD3 + α-CD46, in presence of 10U/mL IL-2. Data are represented as means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Tukey multiple comparison test.

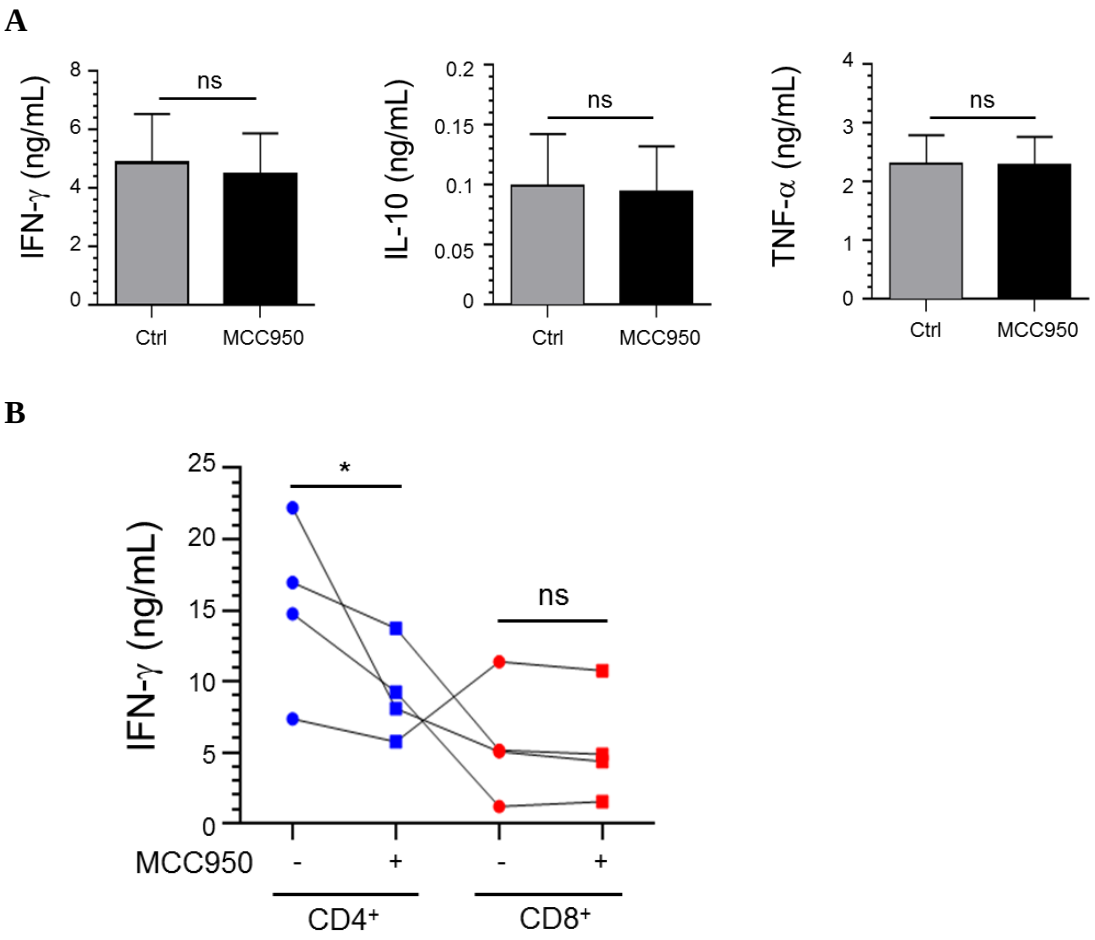


Figure 3.12 Effect of NLRP3 inflammasome inhibition (MCC950) on cytokine secretion by CD8$^+$ T cells

(a) Secreted IFN-γ, IL-10 and TNF-α (ng/mL) by CD8$^+$CD56$^-$ FACS-sorted cells from HDs, activated *in vitro* (60 hours) with α-CD3 + α-CD46, in presence of MCC950 (10 μM) or vehicle control (n=8). (b) Secreted IFN-γ by CD4$^+$CD56$^-$ versus CD8$^+$CD56$^-$ FACS-sorted T cells from the same HDs (n=4), activated *in vitro* (60 hours) with α-CD3 + α-CD46, in presence of MCC950 (+) or vehicle control (-). Data are represented as means ± SEM, *P < 0.05, ns = not significant, paired Student's t test.

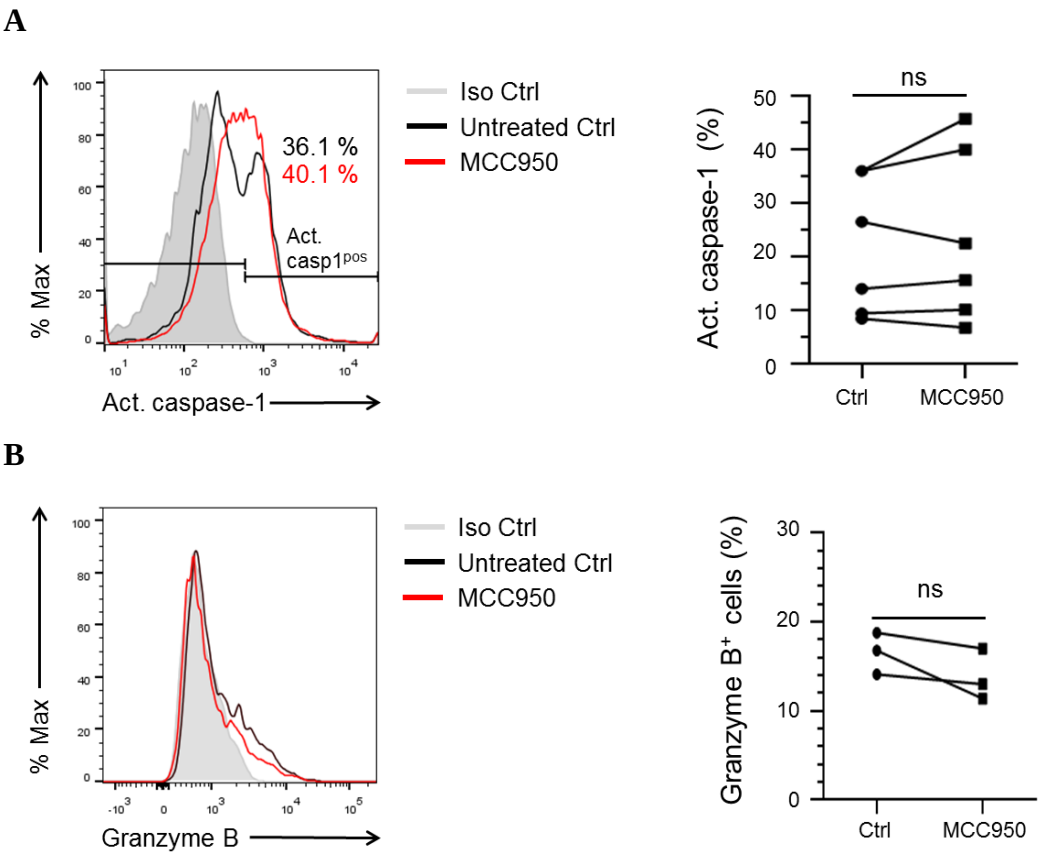


Figure 3.13 Effect of NLRP3 inflammasome inhibition on caspase-1 activation and degranulation in CD8$^+$ T cells

Flow cytometric staining for activated caspase-1 (cleaved caspase-1 measured by FLICA staining assay) (a) and intracellular granzyme B (b) on CD8$^+$CD56$^-$ FACS-sorted cells from HDs, activated *in vitro* (60 hours) with α-CD3 + α-CD46, in presence of MCC950 (10 μM) or vehicle control. Data are represented as means ± SEM, n=6 (a) and n=3 (b). ns = not significant, paired Student's *t* test.

3.5 Discussion

In the remaining part of these PhD studies, preliminary data have been produced about the involvement of complement CD46 and C5-mediated signals during activation and induction of effector functions of human $CD8^+$ cytotoxic T cells. Importantly, in these studies, only $CD8^+CD56^-$ T cells were included to exclude any effects derived from 'contaminating' $CD8^+CD56^+$ natural killer T cells that can also produce IFN-γ and have cytotoxic activity (Godfrey *et al*., 2004).

As expected from previous studies (Astier *et al*., 2000), CD46 expression has been observed at mRNA and protein level also in $CD8^+$ T lymphocytes (Figure 3.3). Not significant differences in total CD46 protein expression have been observed between $CD4^+$ and $CD8^+$ cells from the peripheral blood of the same HDs, although previous studies reported higher extracellular CD46 expression in $CD8^+$ lymphocytes (Christmas *et al*., 2006). In line with this latter observation, higher expression of the transcripts for CD46 isoforms bearing both tail 1 and tail 2 was detected in $CD8^+$ T cells from two out of three healthy donors when compared with $CD4^+$ cells (Figure 3.3B). However, only further studies on an enlarged group of HDs will help to evaluate difference in CD46 expression among different lymphocytic subsets and its biological significance. In addition, it may be helpful to also sort the $CD8^+$ T cells into naïve and memory cells and use resting and α-CD3, α-CD3 + α-CD28 and α-CD3 + α-CD46-activated T cells for the analysis of CD46 isoforms in the future to potentially observe differences in CD46 isoform expression between $CD4^+$ and $CD8^+$ T cells.

Importantly, *in vitro* co-stimulation with CD46 leads to a stronger $CD8^+$ T cell activation when compared to anti-CD3 stimulation either alone or with anti-CD28 co-stimuli: anti-CD3 + anti-CD46 treatment results in increased $CD8^+$ T lymphocytes blasting (not showed), increased IFN-γ and TNFα secretion (Figure 3.4) and degranulation, as assessed by surface-expressed LAMP1 and intracellular produced granzyme B (Figure 3.5). Also increased perforin has been observed upon anti-CD3 + anti-CD46 *in vitro* stimulation but the data are not shown here as more

biological replicates are needed to obtain statistical significance. It should be noted here, that the measurement of intracellular C3 and intracellular C3a and C3b generation as well as the definition of C3aR expression in resting and activated $CD8^+$ T cells is currently missing but underway. Also, cathepsin L cleaves intracellular C3 into bioactive C3a and C3b in $CD4^+$ T cells (Liszewski *et al.*, 2013) and it will be interesting to assess if cathepsin L plays a similar role in $CD8^+$ T cell C3 activation. Further, to better assess the functions of CD46, C3aR and C5aR1, it has been envisaged the use of the CRISPR-Cas9 technique (Sander and Joung, 2014) to generate $CD4^+$ T cells and $CD8^+$ T cells deficient in these molecules.

Interestingly, previous work by Kickler and colleagues (Kickler *et al*, 2012) reported no differences in activation and IFN-γ secretion by $CD8^+$ T cells upon CD46 *in vitro* co-stimulation when compared with anti-CD3 stimuli alone at a similar time point post activation (72 hours). Nevertheless, this group used a different anti-CD46 antibody clone and they did not add IL-2 to cultures, and it is known that signaling coming from IL-2 receptor co-operates with CD46 activity in sustaining $CD4^+$ T cells responses (Cardone *et al.*, 2010). In the future, titration of the signal coming from the anti-CD3 stimulation (different concentrations of immobilised antibody) or of IL-2 or IL-15 (various cytokines concentration into culture) might help to address what are the further signals required by CD46 co-stimulation in enhancing $CD8^+$ lymphocytes effector responses.

The major functional difference of CD46 activation between $CD4^+$ and $CD8^+$ T cells is that no substantial IL-10 secretion occurs from *in vitro* activated $CD8^+$ T cells upon CD46 stimulation (Figure 3.4). This is in line with previous reports (Kickler *et al.*, 2012), and although CD46 co-stimulation results in small detectable levels of this cytokine, it is about 100 fold lower than the amount of IL-10 secreted by $CD4^+$ T cells from the same donor controls. The different levels of IL-10 production might depend on a different expression of the CD46 CYT-1 and CYT-2 isoforms in $CD8^+$ T cells when compared with $CD4^+$ cells, as it is known that CD46 tail 1 and tail 2 have different roles in regulating Th1 induction and contraction in $CD4^+$ lymphocytes (Cardone *et al.*, 2010). Expression studies on three healthy donors resting $CD4^+$ and $CD8^+$ cells did not demonstrate differences in the proportion of

expression of both CD46 tails (Figure 3.3). However the mRNA and protein expression analysis of *in vitro* activated $CD8^+$ versus $CD4^+$ T lymphocytes of the same healthy donor (possibly at different time points of cell activation) is likely more important for the interpretation of such functionally different outcomes. It should also be noted that the intracellular domains of CD46 are cleaved by γ-secretase upon stimulation in several cell types (Weyand *et al*., 2010) and translocate to the nucleus (Kolev *et al*., 2015). Thus, an analysis of nuclear CYT-1 and CYT-2 with specific antibodies generated by the Kemper laboratory will also be included in the future.

In accordance with the increased granzyme B (and perforin) expression and degranulation, activation of $CD8^+$ T cell by CD46 co-stimulation results in heightened killing ability towards "antigen presenting cells", such as the DU145 cells, a prostate cancer cell line, demonstrated by the increased apoptosis and necrosis of cancer cells (Figure 3.6). However, these experiments needs to be repeated with T cells from different donors and to obtain statistical significance as the 'cytotoxic activity' among $CD8^+$ T cells could vary among donors, depending on the specificity of their TCR toward the tumor cell line antigens. This cytotoxicity assay will also allow in future to assess the ability for complement or inflammasome regulators, co-incubated with $CD8^+$ cells during *in vitro* activation, to modulate $CD8^+$ lymphocytes killing ability.

In similitude to the C3 and CD46 system, $CD8^+$ T cells appear to have also the C5 system with expression of the C5a receptors C5aR1 and C5aR2 similar to $CD4^+$ T cells: with cytoplasmic expression of both receptors, while only C5aR2 and not C5aR1 protein was detectable on the cell surface in resting cells (Figure 3.7). This is in contrast to observations by Raedler and colleagues (Raedler *et al*., 2009), who showed evidence for cell surface C5aR1 activity by mouse $CD8^+$ T cells as they can be activated by the addition of C5a to cultures; nonetheless, this group evaluated the effect of C5a T cells activation in co-culture with APCs (thus not accounting for paracrine cytokine production upon C5a binding to APCs in driving T cell responses) and they only evaluated C5aR1 expression at the mRNA and not at the protein level. One novelty of the work performed in this thesis is that there are no

data published with regards to C5aR2 expression in $CD8^+$ T cells, and, in addition, intracellular total C5 protein expression has been demonstrated for the first time in these cells (Figure 3.8). Follow-up experiments should now include the evaluation of autocrine C5a generation by $CD8^+$ T lymphocytes, possibly C5a radioactive binding studies on this cell type (in analogy to the studies performed on $CD4^+$ T cells, Chapter 2), and a thorough assessment of C5aR1 and C5aR2 transcript and protein expression in resting and activated $CD8^+$ T cells.

In line with extracellular expression of C5aR2 by $CD8^+$ T cells, use of a C5aR2 specific peptide agonist (Croker et al., 2016) leads to significantly diminished IFN-γ secretion (Figure 3.9) and degranulation, with significant reduced extracellular LAMP1 and intracellular granzyme B upon anti-CD3 + anti-CD46 *in vitro* activation (Figure 3.10). These data suggest a role for extracellular C5aR2 signaling in negatively regulating $CD8^+$ T cell responses. A better understanding of the involvement of autocrine C5 complement activation in $CD8^+$ lymphocytes effector response would be possible only after in depth evaluation of several C5aR2 agonists/antagonist at different concentrations (dose-dependent affect evaluation by titration) and possibly also C5aR1 agonist/antagonist/siRNA/CRISPR-Cas9 with downstream assessment on IFN-γ production (ROS generation) and cytotoxic activity.

Apart from the complement immune sensors, also the existence of a potential intrinsic NLRP3 inflammasome was investigated in human $CD8^+$ T cells, because: 1) $CD4^+$ T cells have an intracellular NLRP3 inflammasome machinery, which drives IL-1β secretion and sustenance of IFN-γ secretion (Chapter 2); 2) $CD8^+$ T cells have the ability to respond to paracrine IL-1β and IL-18 (Ben-Sasson *et al*., 2013, Soudja *et al.*, 2012). In line with our observations in $CD4^+$ T cells, also $CD8^+$ T lymphocytes express the NLRP3 protein (Figure 3.11), which, similarly to $CD4^+$ cells, increases significantly upon CD46 co-stimulation. Therefore, the existence of an NLRP3 inflammasome within $CD8^+$ cells was interrogated by treating *in vitro* activated $CD8^+CD56^-$ cytotoxic lymphocytes with the NLRP3 specific antagonist MCC950 (Coll et al., 2015). Noteworthy, $CD8^+$ lymphocytes (n=8 healthy donors)

did not show reduced IFN-γ secretion upon NLRP3 inflammasome inhibition (Figure 3.12A), while $CD4^+$ cells from the same healthy donor produced less IFN-γ upon MCC950 treatment (Figure 3.12B) in analogy with previous data (Chapter 2). In addition, MCC950 treatment did not result in reduced caspase-1 cleavage in $CD8^+$ cells activated *in vitro* (Figure 3.13A). These evidences suggest that although NLRP3 is present in $CD8^+$ lymphocytes, it does not participate in the formation of a canonical caspase-1 dependent inflammasome in regulating IFN-γ production by this cell type. Future experiments will measure whether the inhibition of caspase-1 with a specific inhibitor (see Chapter 2) has also no effect on IFN-γ production by $CD8^+$ T cells and whether NLRP3-ASC 'specks' assembly in this cell type following activation. If it is the case, the absence of specks formation and of caspase-1 inhibitory effect would further indicate that the NLRP3 inflammasome does not form in $CD8^+$ T cells and/or is not required for normal IFN-γ activity in this cell type. Of note, caspase-1 can also be activated in an NLRP3 inflammasome independent fashion via TNF-α (Furuoka *et al*., 2016).

Because, it had previously been shown that granzyme B, produced by cytotoxic T and natural killer cells, is another enzyme which stimulates IL-18 maturation (Omoto *et al.*, 2010), it was assessed the possibility that the NLRP3 inflammasome operates together with granzyme B and not caspase-1 to induce autocrine IL-18 production. Notwithstanding, in contrast with this hypothesis, MCC950 treatment did not result in substantial differences in granzyme B expression (Figure 3.13B), but more replicates are needed to obtain statistical significance. Finally, the potential functional granzyme B/NLRP3 interaction can be studied in future by means of colocalization in immunofluorescence experiments or protein co-immunoprecipitation.

In support of the absence of a functional NLRP3 inflammasome in $CD8^+$ T cells, either operating via caspase-1 or granzyme B, neither IL-1β nor IL-18 secretion was detectable by $CD8^+CD56^-$ T cells activated *in vitro* via ELISA (data not shown). Although, it is possible that these cytokines are not detected because they are quickly engaged by their receptors in an autocrine fashion, therefore this possibility should be addressed by measuring the mRNA for these cytokines and by performing intracellular cytokine staining as well as the use of *in vitro* assays which include the

addition of either the IL1RA or IL18BP, which respectively block the IL-1β/IL-1R1 and the IL-18/IL-18R binding in the future.

To further define how the autocrine complement system drives IFN-γ production and cytotoxic activity in an NLRP3 inflammasome-independent fashion in $CD8^+$ T cells, we have performed comparative gene microarray experiments using *in vitro* activated $CD8^+$ T lymphocytes after anti-CD3 alone or anti-CD3 + anti-CD46 stimulation. These data are currently being analysed and hopefully give clues which signaling pathways are driven specifically by CD46 in $CD8^+$ T cells. These array data will also be complemented by gene arrays derived from similarly activated $CD8^+$ T cells isolated from a CD46-deficient patient.

Previous work using mouse T cells indicated already an inflammasome independent role for NLRP3 in T cell activation: in one study (Bruchard *et al*., 2015), it was demonstrated that NLRP3 in mouse $CD4^+$ T cells can translocate to the nucleus and function as transcription factor to regulate *Il4* gene expression and Th2 induction. Thus, NLRP3 in human $CD8^+$ T cells may operate also via an alternative mechanism independent from the formation of an oligomeric inflammasome complex, which can involve nuclear translocation and activity as transcriptional regulator. There is also other work supporting an 'alternative' function for NLRP3. NLRP3, in analogy with other NOD-like receptors, may act as intracellular PAMPs sensor without assembling in an inflammasome machinery: it has been demonstrated that NOD1, another NLR, is able to co-operate with TLR2 and TCR stimulation intrinsically in mouse and human $CD8^+$ lymphocytes to induce their proliferation and effector functions (IFN-γ and TNF-α secretion) (Mercier *et al*., 2012). The author of this study did not report any NLRP3 expression by murine $CD8^+$ T cells, although expression by human cells was not investigated, and there might be species-specific differences for NLRP3 expression in $CD8^+$ lymphocytes. Further experiments to assess this hypothesis include the evaluation of NLRP3 nuclear translocation (confocal microscopy, image stream flow cytometry), and NLRP3 knockdown by siRNA in $CD8^+CD56^-$ lymphocytes. In addition, gene array analyses will be performed using $CD8^+$ T cells from CAPS patients in order to define the role of NLRP3 in $CD8^+$ T cells.

When the data derived from the *in vitro* experiments using $CD8^+$ T cells from HDs, CAPS patients and T cells in which key proteins have been knocked down are conclusive and have pinpointed the role of complement receptors and/or NLRP3 in these cells, additional studies can then be planned which includes suitable *in vivo* mouse models (for example, induced colitis experiments with adoptive transfer of $CD8^+$ T lymphocytes).

Finally, preliminary observations have also been generated showing that bulk $CD8^+$ cells, in contrast to highly pure sorted $CD8^+CD56^-$ cytotoxic T lymphocytes (that are devoid of NKT cells), respond to MCC950 treatment with reduced IFN-γ production after *in vitro* anti-CD3 + anti-CD46 stimulation (data not shown). This indicates that NKT cells, like $CD4^+$ T cells, may produce IFN-γ indeed in a canonical NLRP3 inflammasome-dependent fashion. Thus, in the next set of experiments, NKT cells (specifically the $CD3^+CD8^+CD56^+$ subset) and NK cells (which are $CD3^-CD56^+$) (Godfrey *et al*., 2004; Wang *et al*., 2015), will also be included into a comparative analysis of the NLRP3 function in different cell subsets. The rationale behind this approach is to generate a 'T cell lineage relationship tree' based on the usage (or not) of the new 'complement-inflammasome' axis for effector function. This work complements on-going parallel but independent work in the Kemper laboratory that evaluates the function of intracellular complement in the thymic selection process of T cell lineage development.

In conclusion, as both complement and inflammasome activation products, modulating $CD8^+$ T cell function in a paracrine and/or autocrine fashion, have been demonstrated to profoundly control *in vivo* infection (Fang *et al*., 2007; Soudja *et al*., 2012), transplantation (Vieyra et al., 2011), and cancer (Ghiringhelli *et al*., 2009; Vadrevu *et al.*, 2014) outcome, a better understanding of how complement and inflammasome, operate - intrinsically or not - in human $CD8^+$ T lymphocytes can help in designing therapeutic strategies to target these pathways in infectious, autoimmune and tumor diseases.

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